

Original Paper

Investigating the Inhibitory Effect of Fatty Acids on NMDA Receptor

Tian yun Ma^{1*} & Philip Chen¹

¹ School of Biological Sciences, Royal Holloway, University of London, Egham, UK

* Tian yun Ma, School of Biological Sciences, Royal Holloway, University of London, Egham, UK

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Abstract

*Previous research has showed that AMPA receptor can be used as a molecular target for anti-epileptic drugs, and there is evidence of an inhibitory effect of several fatty acids on AMPA receptor; for example, Decanoic Acid (DA), 4-Ethyl Octanoic Acid (4EOA) and 4-Butylcy Cloheaxne Carboxylic Acid (4BCCA). However, as NMDA receptor and AMPA receptor are quite similar in structures and are widely distributed in CNS, there is a concern on whether the three fatty acids also have an effect on NMDA receptor; and NMDA receptor inhibition may result in some unwanted side effects, such as hallucinations, nightmares, and memory loss. Two-electrode voltage clamp was performed on the oocytes of *Xenopus Laevis* expressing the NMDAR subunit GluN1/N2A, to record agonist evoked inward currents generated from oocytes exposed to different concentrations of fatty acid solutions. On further examination it was found that all of the three acids have some inhibitory effect on NMDA receptor, and seem to act as non-competitive antagonists. The 4EOA has the strongest inhibition effect on NMDA receptor, followed by 4BCCA and DA. However, the three fatty acids only have a clear inhibitory effect when the concentration of the corresponding fatty acid is higher than 500 μ M, which is considered high for clinical use. Moreover, the fatty acids tested had a stronger inhibitory effect on NMDA receptor containing the GluN1/N2B subunits.*

Keywords

AMPA receptor, NMDA receptor, epilepsy, decanoic acid, 4-ethyl octanoic acid, 4-butylcyclohexane carboxylic acid

1. Introduction

1.1 Glutamate

In the nervous system, the proportion of neural glial cells (mainly astrocytes) and neurons is about 10:1. Astrocytes are located between neurons and blood capillaries, which plays an important role in the formation of blood-brain barrier (Brusilow et al., 2010). Under normal conditions, the concentration of glutamate in the cytoplasm of neurons is 5-10mM, in extracellular is 0.2-20 μ M, there is a huge difference between extracellular glutamate concentration and intracellular glutamate concentration (Featherstone, 2010). During synaptic transmission, nerve impulses are transmitted to nerve synapses and nerve endings are depolarized, and synaptic vesicles are released from neurons (i.e., exocytosis) by fusion of synaptic vesicles and plasma membranes. The release of glutamate from vesicles could increase the concentration in synaptic gap from resting 1 μ M to 1.1mM, and maintained peak time is about 1.2ms (Brusilow et al., 2010). All types of glutamate receptors acting on the postsynaptic membrane, will transmit nerve impulses and play a physiological role. Meanwhile, negative feedback regulation is triggered, glutamate uptake by glutamate transporters on astrocytes membranes, as glial cells have strong glutamate uptake ability, and contain glutamine synthetase, which can transform glutamate into glutamine, and then transport into the cytoplasm of presynaptic nerve terminals and produce glutamate by deamination of glutamine (Purves & Williams, 2001). At the same time, some glutamate are catalyzed by glutamate decarboxylase to produce the GABA with inhibition effect (Petroff, 2002). Consequently, glutamate are transported into vesicle lumen by glutamate transporters located on the vesicles, and glutamate are finally stored in the vesicle. In resting, neuron, glutamate is stored in the synaptic vesicles of nerve endings in very small membrane-bound organelles. Therefore a “glutamate-glutamine cycle” is formed between neurons and glial cells (Purves & Williams, 2001).

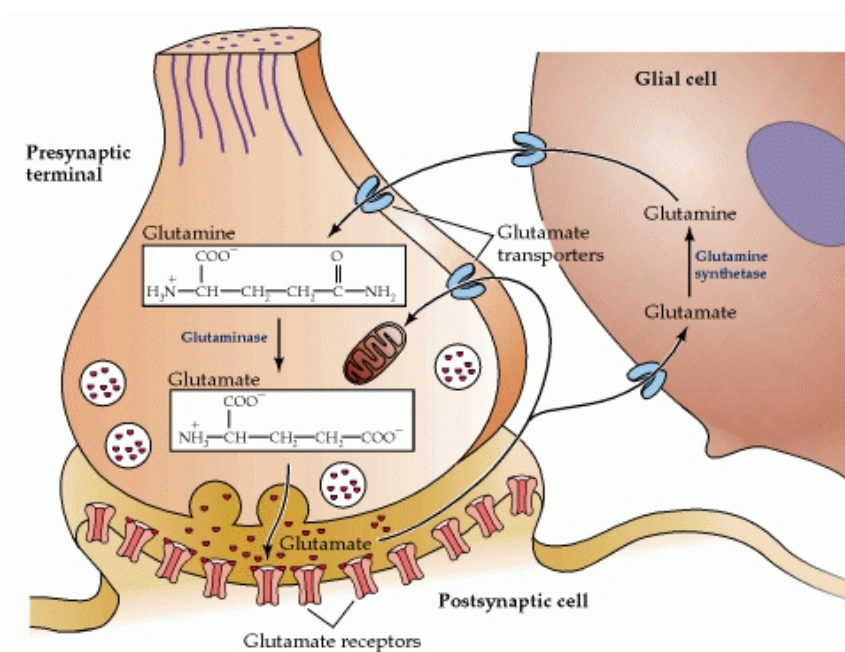


Figure 1. The Glutamate-Glutamine Cycle

Glutamate are removed by the glutamate transporters on both glial cell and presynaptic terminals. Glutamate are converted to glutamine in glial cell by glutamine synthetase, and glutamine are converted to glutamate in presynaptic terminals by glutaminase once they receive glutamine. The supply of glutamate is maintained by the glutamate-glutamine cycle (Purves & Williams, 2001).

Glutamate is a very important neurotransmitter in the brain as they serve for almost all excitatory neurons (Stahl, 1997). There are 2 main categories of glutamate receptors, which are ionotropic glutamate receptors and metabotropic glutamate receptors. Ionotropic glutamate receptors fall into 3 types of receptors, which are NMDA receptor, AMPA receptor and kainate receptor.

1.2 AMPA Receptor

AMPA receptor dysfunction can lead to a variety of neurological damage and mental disorders. The structure and function of AMPA receptor, and the mechanism of epilepsy is reviewed.

AMPA receptor is a tetramer composed of 4 subunits GluA1-GluA4 (also called GluR1-GluR4), which originated from the synthesis of rough endoplasmic reticulum, each subunit has 1 large N-terminus, 3 transmembrane domains, 1 transmembrane ion pore and 1 C-terminus (Shi, 1999). GluA2 always makes the AMPA receptors impermeable to Ca^{2+} , however, AMPA receptors are highly permeable to Ca^{2+} if they only composed of GluA1/3/4 (Han et al., 2017).

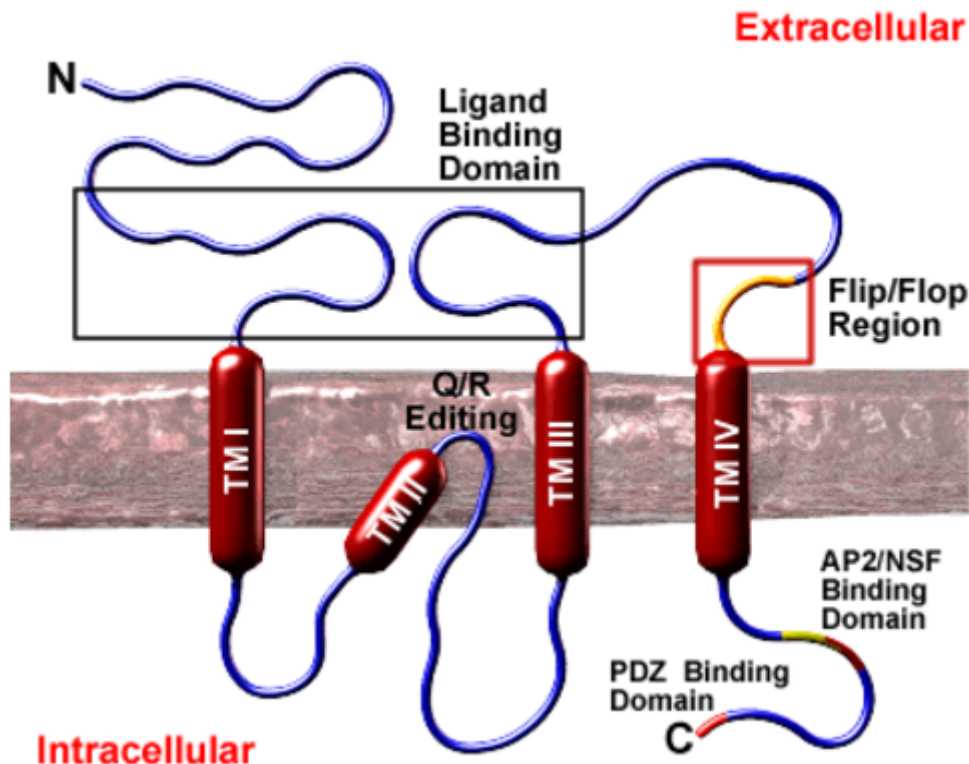


Figure 2. The Structure of GluA2 Subunit of AMPAR

<http://www.bristol.ac.uk/synaptic/receptors/ampar/>

The GluA2 subunit is typically composed of the 3 domains, the extracellular N-terminus and intracellular C-terminus. GluA2 governs the AMPA receptor's permeability to Ca^{2+} .

AMPA receptor is mainly mediated by the central nervous system of the fast excitatory synaptic transmission, and they are essential in synaptic transmission efficiency, neuronal integration and synaptic plasticity (Gouaux, 2004). AMPA receptor is ligand gated ion channel receptor, receptor ion channel opens only when excited, and only monovalent cation can flow through the ion channel, such as K^+ and Na^+ . Influx of extracellular Na^+ leads to depolarization of postsynaptic membrane, inducing rapid EPSP (excitatory postsynaptic current), and then participating in excitatory synaptic transmission (Lajeunesse et al., 2012). At the same time, membrane depolarization resulted from AMPA receptor removes the Mg^{2+} in the channel of NMDA receptor, leads to activation of NMDA receptor. Once NMDA receptor channel opens, the influx of Ca^{2+} will increase the intracellular Ca^{2+} concentration, triggering a series of biochemical reactions, and even results in LTP (long-term potentiation) (Nishiyama & Hanaoka, 2000).

The sudden increase in the excitability of excitatory neurons results an uncontrolled spontaneous abnormal discharge, and it associated with symptoms of recurrent seizures (Chang & Lowenstein, 2003). Over-activation of AMPA receptor may has been associated with acute nerve injury, such as hypoxia-ischemia, hypoglycemia or persistent seizures, and "GluR2 hypothesis" has been suggested:

Down-regulation of GluA2 subunit gene expression in neurons made them more susceptible to toxicity of endogenous glutamate (Pellegrini-Giampietro, 1997). KA (Kainic Acid) has been reported to induced epilepsy in hippocampal CA3 region, and decrease the expression of GluA2, which is subsequently by GluA1 and GluA3 in the AMPA receptor channel. As a result, the permeability of AMPA receptor to Ca^{2+} increases, thus causing damage to hippocampal neurons (Sommer et al., 2001). Moreover, Friedman (2003) demonstrated damage to hippocampal CA3 neurons, but not to CA1 neurons in a rat model of KA-induced epilepsy. In contrast, the injection of AMPAR antagonist 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2, 3-dione (NBQX) exerted protective effects. This has been corroborated by Yamashita et al. (2004) who showed that AMPAR antagonist 2-[N-(4-chlorophenyl)-N-methylamino]-4H-pyrido [3, 2-e]-1, 3-thiazin-4-one (YM928) could also control seizures in KA-induced epilepsy. Similarly, the noncompetitive AMPA receptor antagonist 2, 3-benzodiazepine (GYKI52466) could mitigate the seizures, shorten their duration, and exert an anticonvulsive effect (Rakovska et al., 2002). Based on these previous findings, AMPARs, particularly the GluR2 subunit, are involved in the pathogenesis of epilepsy, while AMPAR antagonists may be candidates for an effective epilepsy treatment.

1.3 NMDA Receptor

NMDA receptor is also an ionotropic glutamate receptor, which is mainly involved in the development of neural circuits in the refinement and trigger a variety of forms of synaptic plasticity (Li & Tsien, 2009). In recent years, evidence has shown that the subunits of NMDA receptor have complex physiological and pharmacological properties. The number, distribution and subunit composition of NMDA receptors are not static, it changes in cell specificity and synapse specificity in the process of development and neuronal activity (Paoletti et al., 2013). The bidirectional change of the NMDA receptor is the basis of the remodeling of synaptic plasticity, and the abnormal regulation can lead to the occurrence of mental disorders, such as memory deficits, hallucinations, schizophrenia, and so on (Paoletti et al., 2013).

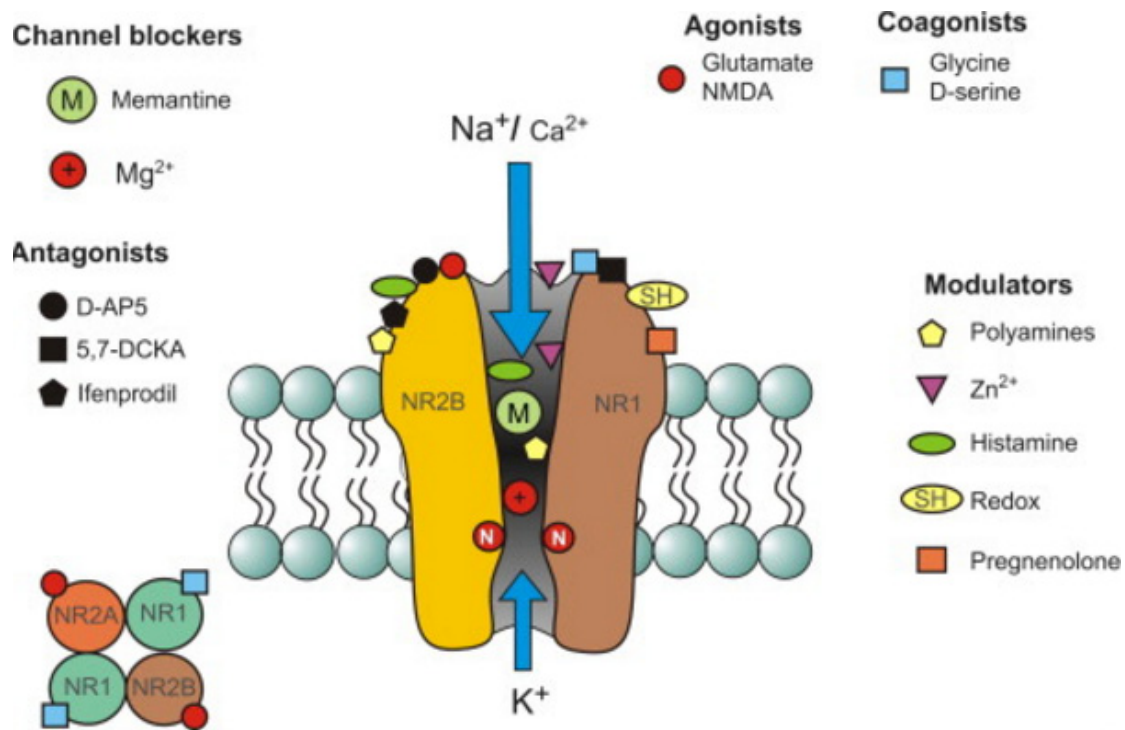


Figure 3. The Structure of NMDA Receptor

This NMDA receptor consisting of GluN1 and GluN2 subunit. Both glutamate and glycine are needed to activate the NMDA receptor. The Mg^{2+} will block the NMDAR ion channel at resting membrane potential, only the depolarization of postsynaptic nerve cell will remove the Mg^{2+} ion, and allow the Ca^{2+} current to flow through the NMDA receptor.

The activation requirement of NMDA receptor is special as its ion channel needs 2 kinds of agonists to activate, namely glutamic acid (glutamate) and glycine (Cull-Candy et al., 1998), with the binding site of glutamate lying on GluN2 subunit and binding site of glycine on GluN1 subunit. Researches by Yao (2006) indicated that GluN3 subunit was with high affinity to glycine, which proved that glycine could activate GluN3 subunit.

The density of NMDA receptor subunit in different part of brain tissue is different with significant difference of density at various developmental stages GluN1 subunit was widely distributed in CNS (central nervous system). GluN2B subunit was widely distributed in neonatal brains and GluN2D subunit widely spread in neonatal brain stem. The content of the two gradually fell down with newborn growth and development process. The GluN2A subunit which is mainly located in hippocampus and cortex while GluN2C subunit was rich in cerebellum (Cull-Candy et al., 1998). By observing the ultra structure of NMDA receptor, people such as Radley et al. (2007) found that GluN2B subunit mainly located in thalamus and amygdala of adult brains.

GluN3A mRNA was expressed most in the cerebral cortex of 1 week newborn baby and then its expression fell down. By applying ISH (In Situ Hybridization) and immune his to chemistry method,

Chatterton et al. (2002) found that GluN3A subunit was widely distributed in central nervous system while GluN3B subunit mainly could be seen in medulla spinalis and the motor neuron in brain stem. NMDA receptor is a unique ligand-gated ion channel as its activation needs both glutamate and glycine. Normally NMDA receptor consists of two GluN1 subunits and GluN2 subunits is highly permeable to Ca^{2+} . Meanwhile, NMDA receptor's high affinity to endogenous channel blocker Mg^{2+} makes it have voltage dependent blocking characteristics (VanDongen, 2009). Thus, the cell signaling induced by NMDA receptor played an important role in neuronal growth and regulation, neuronal dendrite and axon structure development, synaptic plasticity, learning and memory, etc. Liu et al. (2007) used Western blotting method and found that on cerebral cortical neurons, the NMDA receptor containing GluN2A subunit on the synapse and outside of the synapse could facilitate neuronal growth and protect neuronal damage.

Under physiological conditions, with postsynaptic membrane staying in resting stage, the presence of Mg^{2+} ion in NMDA receptor ion channel can block Ca^{2+} influx as the ion channel gate is voltage dependent. Due to high permeability of GluN2 subunit to Ca^{2+} , the activation of NMDA receptor by endogenous glutamic acid can lead to Ca^{2+} influx and Ca^{2+} will mediate a series of cell signaling pathways. When the amount of Ca^{2+} in postsynaptic neuron cytoplasm greatly increases, the postsynaptic membranes will be depolarized and action potential will be generated by postsynaptic neurons, all of these will lead to LTP (Long Term Potentiation). LTP long-term enhancing synaptic transmission efficiency is considered as the basis of learning and memory, while LTD (Long Term Depression) will reduce synaptic transmission efficiency (TSUMOTO, 1992). In rat hippocampal slices, Berberich et al. (2007) found that following application of high frequency stimulation, GluN2A and GluN2B worked together to facilitate generation of LTP; While using low frequency stimulation, either GluN2A or GluN2B subunit could induce LTP generation.

1.4 Aims and Hypothesis

Recently, more researchers focused on the effect of fatty acids on these glutamate receptors. The fatty acids are carboxylic acids with hydrocarbon chain and terminal carboxyl group (Barnes, 1959). Decanoic acid is a saturated fatty acid. 4EOA and BCCA have been chemically synthesized as novel compounds based on the structure of DA.

Chang et al. (2016) suggested that there was inhibitory effect of Decanoic Acid (DA) on AMPA receptor, with $\text{IC}_{50}=1.16\text{mM}$ on GluA1/2 and $\text{IC}_{50}=0.52\text{mM}$ on GluA2/3. It stated that DA (Decanoic Acid) acted as a non-competitive antagonist on AMPA receptor, and DA may help to control seizure through direct AMPA receptor inhibition. 4EOA (4-Ethyl-octanoic Acid) also showed an inhibitory effect on AMPA receptor, with IC_{50} around 0.65mM on GluA2/A3. 4BCCA (4-Butylcyclohexane Carboxylic Acid) did an inhibitory effect on AMPA Receptor as well, with $\text{IC}_{50}=1\text{mM}$ for GluA1/2, and $\text{IC}_{50}=0.5\text{mM}$ for GluA2/3.

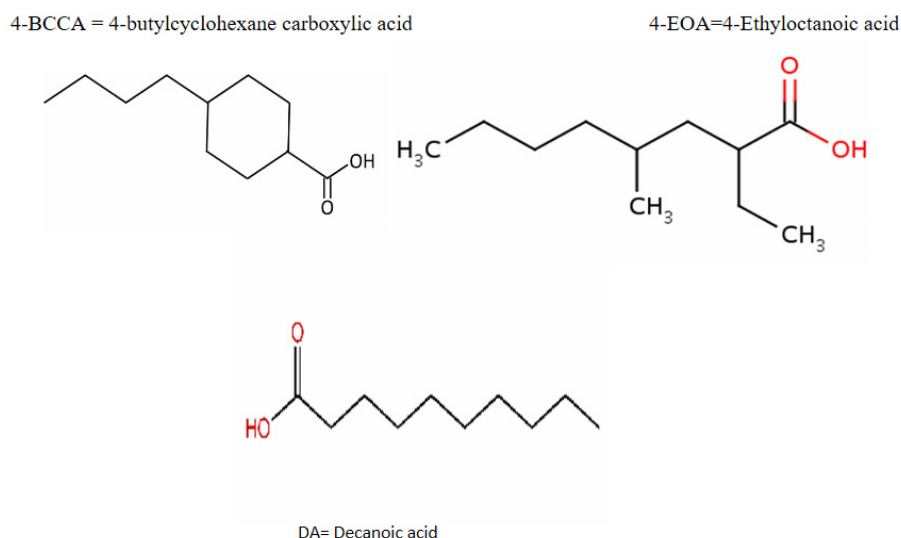


Figure 4. The Structure of the 3 Fatty Acids Used in the Experiment

The left top is 4BCCA (4-Butylcyclohexane Carboxylic Acid), the right top is 4EOA (4-Ethyloctanoic Acid) and the bottom one is DA (Decanoic Acid).

As NMDA receptor is essential for normal CNS function, disruption of NMDA receptor will affect CNS adversely, with the symptoms of memory loss, breathing and locomotion deficits, hallucinations and nightmares (VanDongen, 2009). Therefore, there is a concern arisen on whether inhibition of AMPA receptor during epilepsy treatment will in turn affect the NMDA receptor, since both AMPA receptor and NMDA receptor are glutamate receptors and they are somehow similar in structure and distribution. The aim of this experiment was to investigate whether the 3 acids mentioned before have any inhibitory effect on NMDA receptor, to avoid the side effects when they used as AMPA receptor antagonist for epilepsy treatment, and to examine the sensitivity of GluN2A and GluN2B subunits to these fatty acids respectively. The null hypothesis was there was no any inhibitory effect resulted from the 3 fatty acids on NMDA receptor, and no difference between sensitivity of GluN2A to the fatty acids and sensitivity of GluN2B. The alternative hypothesis was that there was an inhibitory effect of the fatty acids on the resulted from the 3 fatty acids on NMDA receptor, and there was a difference between sensitivity of GluN2A to the fatty acids and sensitivity of GluN2B. The null hypothesis was retained when $p > 0.05$, and the null hypothesis was rejected when $p < 0.05$.

2. Method

2.1 Peeling of the Oocytes and RNA Injection

The *Xenopus laevis* are considered ideal for studying the expression of ion channels and receptors due to their versatile expression system (Bros, 2010). Thus, we used the oocytes of *X. laevis* to express NMDA receptors.

First, the thin membrane composed of blood capillaries covering the oocytes was removed using forceps under the microscope in order to facilitate the RNA injection. To inhibit the release of glutamate from the oocytes, which would increase their death, the oocytes were incubated in 24-well plates with 1X Bath's solution (88 mM NaCl, 1 mM KCl, 2.3 mM NaHCO₃, 15 mM Tris HCl, 0.82 mM MgCl₂, 0.77 mM CaCl₂ with pH adjusted to 7.4 by NaOH [4 M], added to 50 IU/mL penicillin and 50 µg/mL streptomycin) containing 500 µM of (2R)-Amino-5-Phosphonovaleric Acid (APV), which is a glutamate antagonist. A total of 2 mL per well were used for the incubation.

The RNA was stored at -79 °C until used. A total of 2 µL of a mixture of NMDAR subunits GluN1 and GluN2A RNA (the ratio of GluN1: GluN2A is 1:1, the concentration of RNA used was 5 ng/µL) was used for each set of experiment. A total of 46 nL of RNA were injected into one oocyte through the injection pipette (Harvard Apparatus catalogue NO.690133) cut by an electrode puller (Narashinge). Each injected oocyte was placed in an individual well, and the plate was incubated at -15 °C for 1-2 days to allow the RNA to express the NMDA receptors.

2.2 Preparation of the Solutions and Fatty Acids

Glycine and glutamate were used at 50 mM and 100 mM, respectively. A total of 1.72 g of DA/4EOA (molecular weight, 172 g/mol) were diluted with 10 mL Dimethyl Sulfoxide (DMSO) resulting in 1 M DA/4EOA solution. The 1M 4BCCA was previously prepared by others. Ringer's solution (1X) was diluted from 10X Ringer's stock solution (1.15 M NaCl, 25 mM KCl, 100 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]). Given that NMDARs are pH-sensitive, the pH was adjusted to 7.35 with 4 M NaOH. Subsequently, to enable a smoother response current and reduce the current fluctuation, 1.8 mM of BaCl₂ were added to every 1L 1X Ringer's solution. The stock concentration of the fatty acids included in 4BCCA, DA and 4 EOA was 1 M.

2.3 Two Electrode Voltage Clamp (TEVC)

During the electrophysiological measurements, the injected oocyte was removed from the plate each time and placed in the recording chamber immersed with buffer (mixture of Ringer's/glutamate/glycine solution) to acquire the electrophysiological recordings (Axon Instrument DIGIDATA 1322A). The electrodes were made from borosilicate glass (GC150TF7.5, Harvard Apparatus) and generated by the electrode puller (Narashinge). Electrodes were filled up with 300 mM KCl, and the voltage was set to -40 mV. The data were only recorded when the current produced by the oocytes was less than 0.60 µA. Eight tubes linked to the recording chamber were refilled with different solutions and were controlled by a valve commander (ALA Scientific Instruments). Data were subsequently processed in WinEDR software v3.7.5 (University of Strathclyde Glasgow, UK).

2.4 Wild-Type GluN2A Dose Response Curve (Control)

Fresh 1X Ringer's solution was added to the first tube linked to the recording chamber. In the second tube, 100 μ L Ringer's were mixed with 0.1 μ M glutamate and 50 μ M glutamate. The glutamate concentrations in tubes 3 to 8 were 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, 30 μ M and 100 μ M, respectively. The concentration of glycine remained constant. The current at each glutamate concentration was measured. The difference of the response current was calculated between the baseline and each glutamate concentration, and was presented as a percentage to the maximum response. Approximately 6-10 oocytes were randomly selected from the oocytes pool at 2 weeks to record the current for one complete set of wild-type GluN2A dose response curve. A dose response curve was plotted as the normalized response (%) against the glutamate concentration (μ M) by using Igor Pro software v6.1 (WaveMetrics).

2.5 The Calculation of the Half Maximal Effective Concentration (EC50) Curves

2.5.1 The EC₅₀ Curve for 4BCCA

The glycine/glutamate/1X Ringer's solution was mixed with 1 mM 4BCCA and placed into tubes to examine the EC₅₀ curve. The last tube was devoid of 4BCCA to compare the difference in the current recorded with and without fatty acid. A total of 6-10 oocytes were taken randomly from the oocytes pool at 2 weeks to record the current for one complete set of 1 mM 4BCCA EC₅₀ curve experiment. The dose response curve was plotted in the same way for the control. The EC₅₀ value was calculated by using the Igor Pro software v6.1.

2.5.2 The EC₅₀ Curve for DA

The same above-mentioned procedure was repeated by using 500 μ M DA, 750 μ M DA and 1 mM DA.

2.5.3 The EC₅₀ Curve for 4EOA

The same above-mentioned procedure was repeated by using 500 μ M 4EOA, 1 mM 4EOA, 2 mM 4EOA and 3 mM 4EOA.

2.6 The Half Maximal Inhibitory Concentration (IC₅₀) Curve for 4EOA at 3 μ M Glutamate

The glycine/glutamate/1X Ringer's solution was supplemented with 3 μ M glutamate and 50 μ M glycine, and the mixture was placed in tube 2-6 with 4EOA at 100 μ M, 300 μ M, 1 mM, 3 mM and 5 mM, respectively. The IC₅₀ value was calculated by using the Igor Pro software v6.1.

2.7 The DMSO Control

For the DMSO control recording, 100 μ L DMSO replaced the fatty acid in the glycine/glutamate/1X Ringer's solution. The aim behind performing the DMSO control was to verify whether there was any difference between 1 mM fatty acid dissolved in DMSO and DMSO alone, and to ensure that DMSO had no effect on the NMDA receptors.

2.8 The GluN1/N2B NMDA Receptor Subunits

Instead of mixture of GluN1 and GluN2A, 2 μ L of mixture of GluN1 and GluN2B subunits were injected into the peeled oocytes (the ratio of GluN1: GluN2B is 1:1, the concentration of RNA used was 5 ng/ μ L). The same measurements as for the AMPA receptor subunits were performed, including

the control, 4BCCA EC50 curve (100 μ M and 1 mM 4BCCA), DA EC50 curve (500 μ M and 1 mM DA), 4EOA EC50 curve (200 μ M and 3 mM 4EOA) on the oocytes with GluN1/N2B subunits.

2.9 Statistical Analysis

The electrophysiological recording data were arranged using Excel 2013. All data we analyzed with SPSS software using paired and unpaired t-test. Statistical significance was considered at $p < 0.05$. When $p < 0.05$, the null hypothesis was rejected. When $p > 0.05$, null hypothesis was accepted.

3. Result

After TEVC (Two Electrode Voltage Clamp) had been performed on the oocytes of *Xenopus laevis*, a electrophysiological recording was obtained to indicate the effect of glutamate on NMDA receptor. 4BCCA was applied first on the oocytes of *Xenopus laevis*, as 4BCCA was available in stock first at the beginning of the experiment. Also, 4BCCA had the lowest IC50 on AMPA receptor (GluA2/3) among the 3 fatty acids we are going to test, and it would be easier to compare the effect of 4BCCA with the other two.

3.1 The Effect of Different Concentrations of 4BCCA on NMDA Receptor

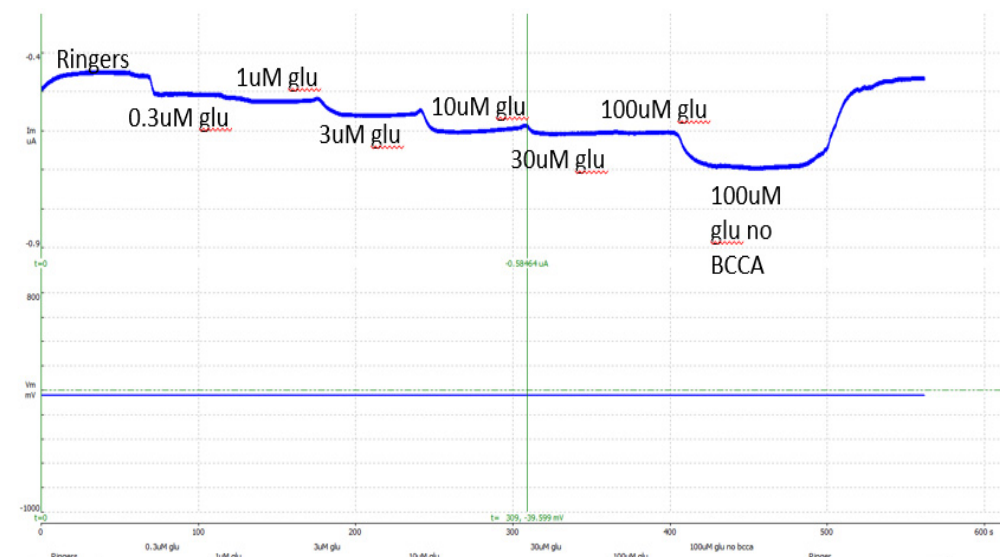


Figure 5. Example of Standard Electrophysiological Recordings for the Effect of 1mM 4BCCA on NMDA Receptor (GluN1/2A)

Glycine concentration was kept at 50 μ M, glutamate concentration varied from 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 100 μ M. 4BCCA concentration was 1mM. The effect of 4BCCA was investigated by comparing 100 μ M glutamate with 1mM 4BCCA and without any 4BCCA.

The effect of 1mM 4BCCA was indicated by a reduction in the current. It was compared latterly with the standard electrophysiological recording (without any addition of fatty acids) to show the difference

the two recordings, and therefore, to get the glutamate dose response curve for each one, and in the end to conclude the effect of 1mM 4BCCA by their normalised response at 100 μ M glutamate and EC50 value. When examine the effect of 1mM 4BCCA on gluN2A and make the glutamate dose response curve to get the EC50 value, the 1mM 4BCCA glutamate dose response curve was not only compared with the control glutamate dose response curve (named control for N2A), but also compare with the DMSO control glutamate dose response curve (named DMSO control for N2A), where DMSO is used for preparing and diluting the fatty acid. Compare the EC50 value and normalised response at 100 μ M glutamate between 1mM 4BCCA and DMSO control could help conclude if the fatty acid itself could be the independent variable that exert an inhibitory effect on NMDA receptor (GluN1/GluN2A), or the DMSO solution could also had an inhibitory effect on NMDA receptor.

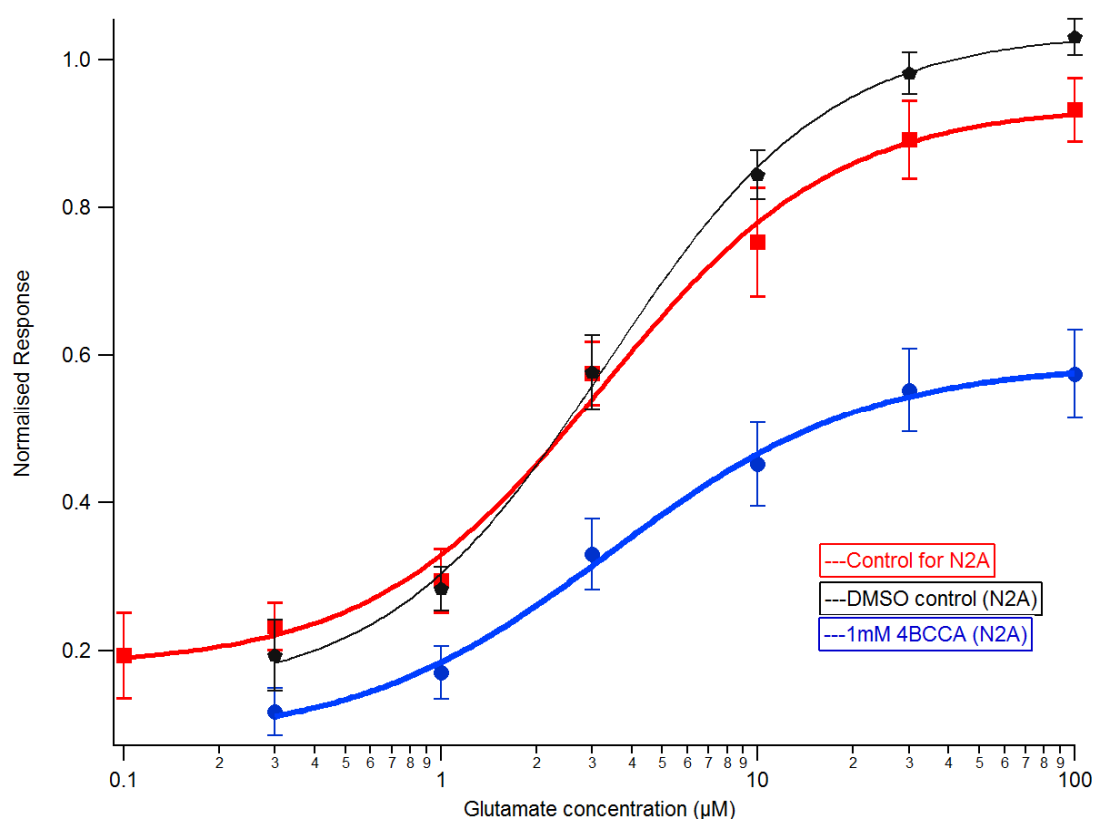


Figure 6. The Effect of DMSO Control and 1mM 4BCCA NMDARs (GluN1/N2A Subunits)

The DMSO solution used for making the fatty acid solutions were examined on whether they had any effect on the normalised response. The volume of DMSO solution added for making this dose response curve was same as the amount of DMSO in 1mM 4BCCA. The error bars were \pm SEM. N=6-10.

The DMSO solution itself had no effect on NMDA receptor (GluN1/N2A), whether on the maximum normalised response at 100 μ M glutamate ($p=0.079$), or EC50 ($p=0.599$). However, the 1mM 4BCCA made an inhibitory effect on NMDA receptor (GluN1/N2A) (for the details see next figure), and therefore the inhibitory effect was completely due to the presence of the fatty acid but not the DMSO

solution.

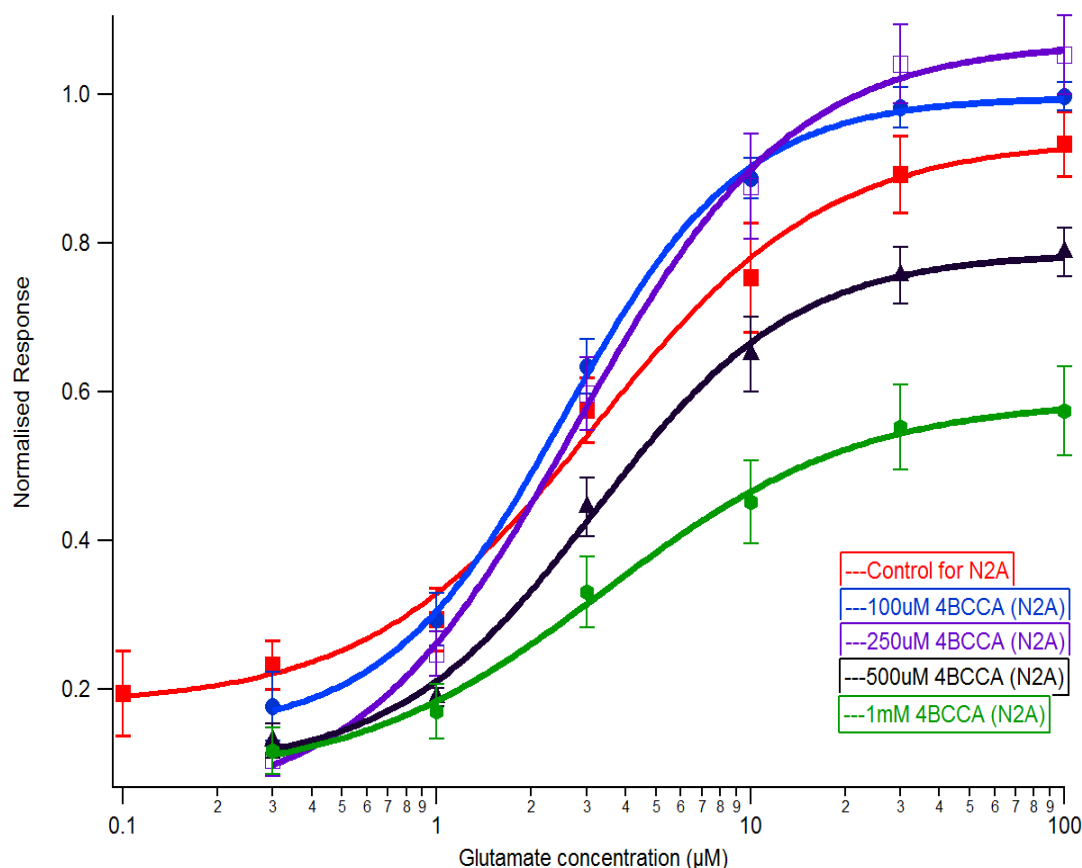


Figure 7. The Effect of Different Concentrations of 4BCCA on NMDA Receptor (GluN1/N2A Subunits)

The single dose response curve of 100µM, 250µM, 500µM, and 1mM 4BCCA at 0.3µM, 1µM, 3mM, 10µM, 30µM, 100µM glutamate compared to the wild type GluN2A (control). The error bars were \pm SEM. N=6-10.

In fact, 4 different concentrations of 4BCCA were used to test the inhibitory effect of 4BCCA on GluN1/N2A subunits, from 100µM, 250µM, 500µM to 1mM. The lowest concentration of 4BCCA applied was determined by the point which resulted in no change in the normalised response at 100µM glutamate, and the highest concentration of 4BCCA applied was determined by the solubility of the fatty acid, as the 4BCCA solution could not be used when there was a precipitate formation.

Concentration of 4BCCA (μM)	Normalised response at 100 μM glutamate	Rate	EC50 (μM)
0	0.93310 ± 0.0429	1.188 ± 0.237	3.2607 ± 0.554
100	0.99671 ± 0.0188	1.5291 ± 0.162	2.5433 ± 0.182
250	1.0523 ± 0.0532	1.2583 ± 0.183	2.7796 ± 0.327
500	0.78728 ± 0.0328	1.3621 ± 0.253	3.1988 ± 0.445
1000	0.57449 ± 0.0601	1.0728 ± 0.238	3.4386 ± 0.68

Figure 8. The Comparison between Wild Type (GluN2A) and Difference Concentrations of 4BCCA on Normalised Response at 100 μM Glutamate (μM), Rate and EC50 (μM) (\pm SEM)

The 0 μM 4BCCA represented the wild type (GluN2A).

Both 100 μM and 250 μM 4BCCA had no any effect on NMDARs (GluN1/N2A) with $p=0.205$ and $p=0.136$ respectively. 500 μM 4BCCA reduced the response at 100 μM glutamate by 15.6% ($p=0.017$). 1mM showed the strongest inhibitory effect and reduced the response at 100 μM by 38.4% ($p<0.001$). Therefore, only 500 μM 4BCCA and 1mM 4BCCA showed inhibitory effect on NMDARs (GluN1/N2A subunits), and the inhibitory effect became stronger when the concentration of 4BCCA increased. There was no change in the EC50 when comparing different concentrations of 4BCCA and wild type (GluN2A). The EC50 showed no significant change between different concentrations of 4BCCA and control (GluN2A) as $p>0.05$ for all the four concentrations.

After trying GluN1/N2A subunit, the effect of 4BCCA was also investigated on GluN1/N2B subunits, to further illustrate the sensitivity of Glu2A and GluN2B subunits to 4BCCA, as the NMDA receptors in CNS contains both GluN1/N2A and GluN1/N2B.

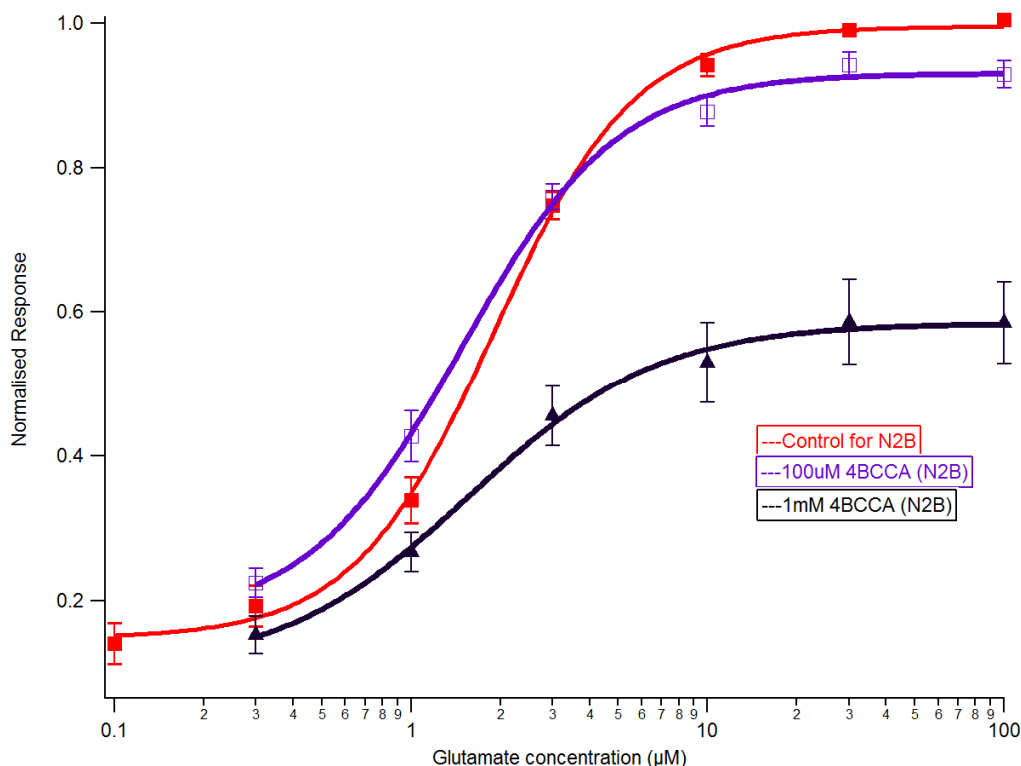


Figure 9. The Effect of Different Concentrations of 4BCCA on NMDA Receptor (GluN1/N2B Subunits)

The single dose response curve of 100μM and 1mM 4BCCA at 0.3μM, 1μM, 3mM, 10μM, 30μM, 100μM glutamate compared to the wild type GluN2B (control). The error bars were \pm SEM. N=6-10.

Concentration of 4BCCA (μM)	Normalised response at 100μM glutamate	Rate	EC50 (μM)
0	1.0050 ± 0.0047	1.827 ± 0.147	1.8998 ± 0.0974
100	0.92963 ± 0.0192	1.6613 ± 0.233	1.4894 ± 0.151
1000	0.58529 ± 0.0565	1.355 ± 0.304	1.5687 ± 0.307

Figure 10. The Comparison between Wild Type (GluN2B) and Difference Concentrations of 4BCCA on Normalised Response at 100μM Glutamate (μM), Rate and EC50 (μM) (\pm SEM)

The 0μM 4BCCA represented the wild type (N2B).

Only the lowest and highest concentration of 4BCCA applied on GluN1/N2A, were used to applied on GluN1/GluN2B, which were 100μM and 1mM 4BCCA respectively, as the main aim of this procedure was to investigate the difference in 4BCCA inhibitory effect between GluN1/N2A and GluN1/N2B.

The 100μM 4BCCA slightly decreased the response at 100μM glutamate by 7.5% ($p=0.004$), and 1mM

4BCCA decreased the response at 100 μ M glutamate by 41.8% ($p < 0.001$). The EC₅₀ of 100 μ M 4BCCA showed a decrease of 0.41 μ M compared to the control (GluN2B) ($P = 0.037$), and the EC₅₀ of 1mM 4BCCA showed a decrease of 0.33 μ M compared to the control (GluN2B) ($p = 0.036$).

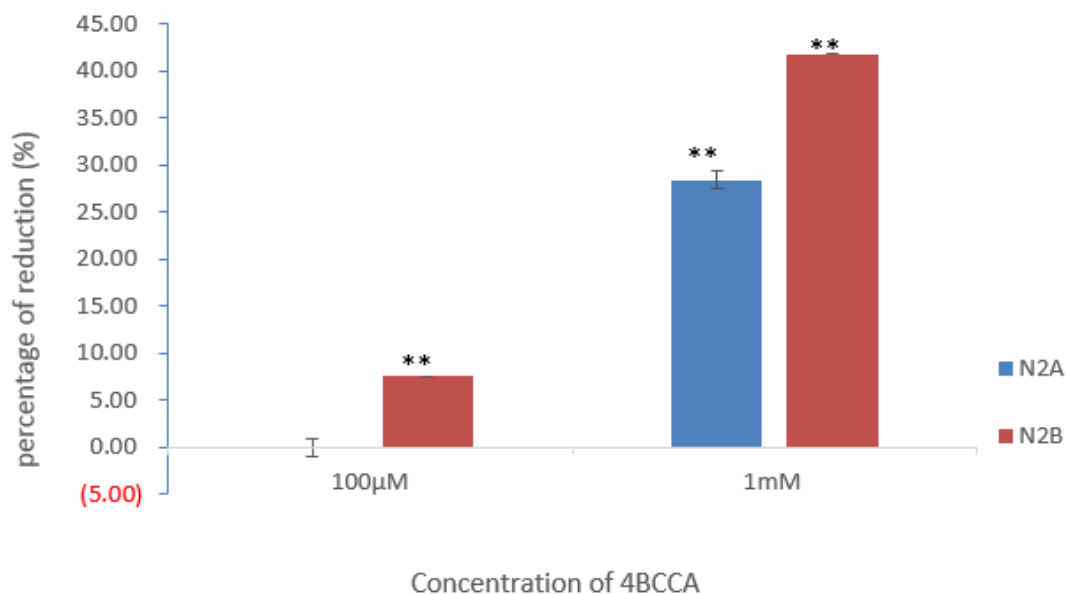


Figure 11. The Comparison of the Percentage of Reduction in Normalised Response for 100 μ M and 1mM 4BCCA between GluN1/N2A and GluN1/N2B Subunits

The difference between normalised response at 100 μ M 4BCCA (GluN2A) was compared to the wild type (GluN2A) to get the percentage of reduction in normalised response for 100 μ M 4BCCA (GluN2A).

** indicated that $p < 0.05$. The error bars were \pm SEM. N=6-10.

There was no significant inhibitory effect of 100 μ M 4BCCA on GluN2A subunit, however, it did show to slightly decrease the normalised response on GluN2B subunit. Moreover, 1mM 4BCCA also showed a stronger inhibitory effect on GluN2B than GluN2A. Therefore, it may concluded that GluN2B was more sensitive than GluN2A subunits to 4BCCA.

3.2 The Effect of Different Concentrations of DA on NMDA Receptor

Since DA and 4BCCA are similar in chemical structure' after doing the 4BCCA glutamate dose response curve, different concentrations of DA were applied on the on the oocytes of *Xenopus laevis* to get the electrophysiological recording, and therefore to plot the DA glutamate dose response curve by similar procedure.

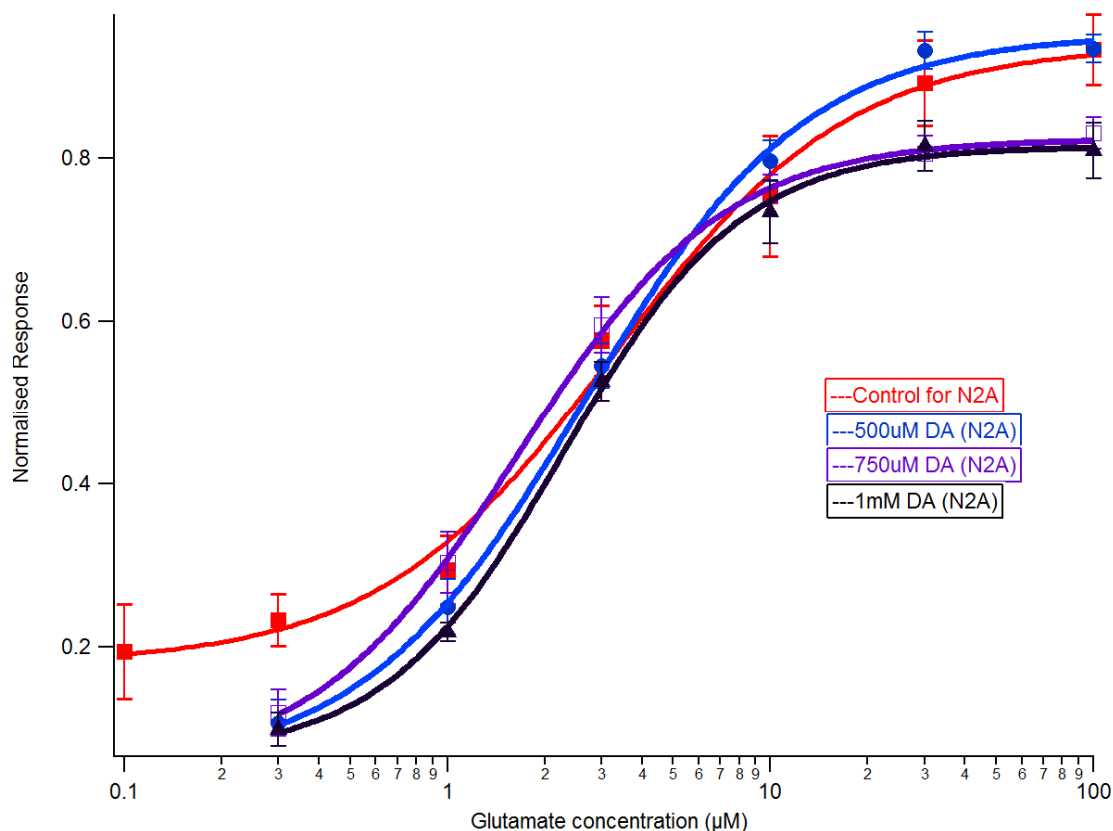


Figure 12. The Effect of Different Concentrations of DA on NMDA Receptor (GluN1/N2A Subunits)

The single dose response curve of 500µM, 750µM and 1mM DA at 0.3µM, 1µM, 3µM, 10µM, 30µM, 100µM glutamate compared to the wild type GluN2A (control). The error bars were \pm SEM. N=6-10. Unlike 4BCCA, there were only 3 different concentrations of DA were used to test the inhibitory effect of 4BCCA on GluN1/N2A subunits, which were 500µM, 750µM and 1mM. The lowest concentration of DA applied was determined by the point which resulted in no change in the normalised response at 100µM glutamate, and the highest concentration of DA applied was determined by the solubility of the fatty acid, as the DA solution could not be used when there was a precipitate formation. The range of DA concentration was smaller compared to 4BCCA, as DA did not result in a decrease of normalised response at 100µM glutamate when it concentration was as high as 500µM.

Concentration of DA (μM)	Normalised response at 100 μM glutamate	Rate	EC50 (μM)
0	0.93310 ± 0.0429	1.188 ± 0.237	3.2607 ± 0.554
500	0.93407 ± 0.0171	1.2688 ± 0.15	2.6463 ± 0.279
750	0.8306 ± 0.0191	1.3726 ± 0.123	1.6558 ± 0.125
1000	0.80937 ± 0.0338	1.577 ± 0.167	2.2984 ± 0.165

Figure 13. The Comparison between Wild Type (GluN2A) and Difference Concentrations of DA on Normalised Response at 100 μM Glutamate(μM), Rate and EC50 (μM) (\pm SEM)

The 0 μM 4BCCA represented the wild type (GluN2A).

Both 500 μM and 750 μM DA had no effect on NMDA receptor (GluN1/N2A) with $p=0.916$ and $p=0.054$ respectively. DA only showed an inhibitory effect when the concentration reached 1mM and the normalised response at 100 μM glutamate was decreased by 13.2% ($p=0.041$). The change in EC50 was not statistical significant at 500 μM DA ($p=0.095$). The EC50 decreased by 49.2% at 750 μM DA ($p=0.002$) and by 29.6% at 1mM DA ($p=0.018$) compared to wild type (GluN2A).

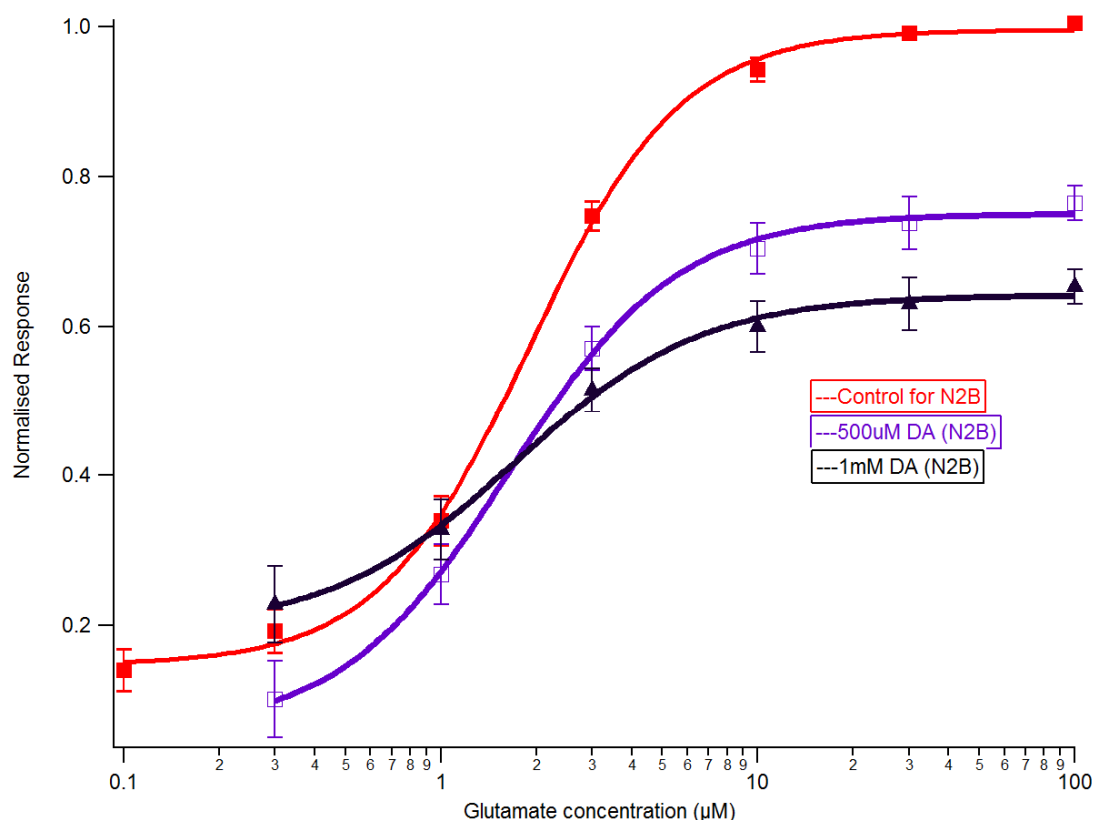


Figure 14. The Effect of Different Concentrations of 4BCCA on NMDARs (GluN1/N2B Subunits)

The single dose response curve of 100 μ M and 1mM 4BCCA at 0.3 μ M, 1 μ M, 3mM, 10 μ M, 30 μ M, 100 μ M glutamate compared to the wild type GluN2B (control). The error bars were \pm SEM. N=6-10.

Concentration of DA (μ M)	Normalised response at 100 μ M glutamate	Rate	EC50 (μ M)
0	1.0050 \pm 0.0047	1.827 \pm 0.147	1.8998 \pm 0.0974
500	0.76428 \pm 0.0164	1.6423 \pm 0.183	1.6437 \pm 0.133
1000	0.65343 \pm 0.0228	1.4776 \pm 0.257	1.7233 \pm 0.235

Figure 15. The Comparison Between Wild Type (GluN2B) and Difference Concentrations of DA on Normalised Response at 100 μ M Glutamate (μ M), Rate and EC50 (μ M) (\pm SEM)

The 0 μ M DA represented the wild type (GluN2B).

The normalised response at 100 μ M glutamate had been decreased by 24.0% at 500 μ M DA ($p < 0.001$) and by 35.0% at 1mM DA ($p < 0.001$) on GluN2B subunit. However, the EC50 did not show any significant change either at 500 μ M DA ($P = 0.243$) or 1mM DA ($P = 0.556$).

Only the lowest and highest concentration of DA applied on GluN1/N2A, were used to applied on GluN1/GluN2B, which were 500 μ M and 1mM DA respectively, as the main aim of this procedure was to investigate the difference in DA inhibitory effect between GluN1/N2A and GluN1/N2B.

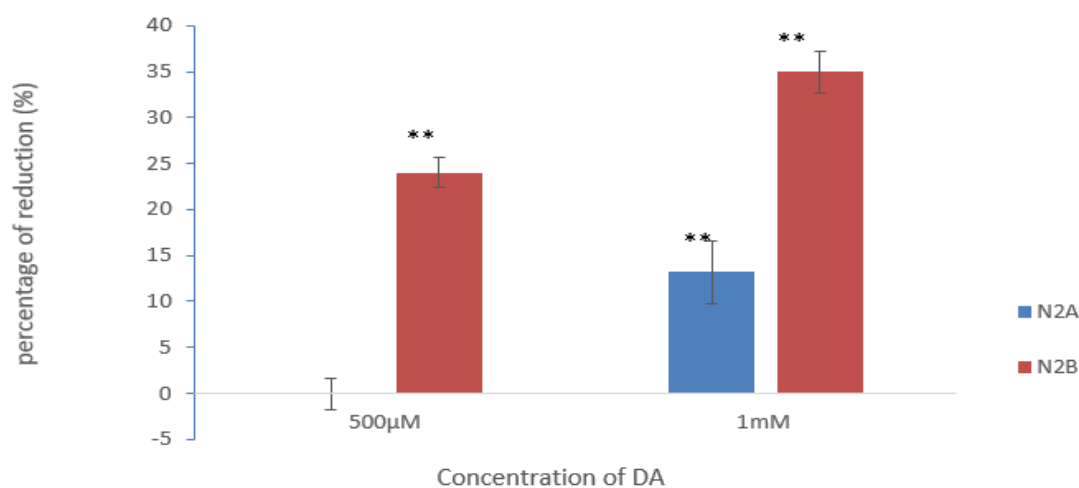


Figure 16. The Comparison of the Percentage of Reduction in Normalised Response for 500 μ M and 1mM DA between GluN1/N2A and GluN1/N2B Subunits

** indicated that $p < 0.05$. The error bars were \pm SEM. N=6-10.

Although 500 μ M DA did not have any inhibitory effect on GluN2A subunits, it did have a relatively strong inhibitory effect on GluN2B with a 35% reduction in normalised response.

Also, 1mM DA only decreased the normalised response by 13.2% on GluN2A, but it decreased the normalised response by 35.0% at GluN2B. In this way, GluN2B was more sensitive to DA than GluN2A. However, it was interesting to find that DA only altered the EC₅₀ when they acted on GluN2A subunits, no significant change in EC₅₀ between DA (GluN2B) and control (GluN2B) found.

3.3 The Effect of Different Concentrations of 4EOA on NMDA Receptor

4EOA was another fatty acid similar to 4BCCA and DA structure, and it had a relatively low IC₅₀ on AMPA receptor (GluA2/3 subunits). The last fatty acid used in this experiment used was 4EOA. Different concentrations of 4EOA were applied on the on the oocytes of *Xenopus laevis* to get the electrophysiological recording, and therefore to plot the 4EOA glutamate dose response curve by similar procedure.

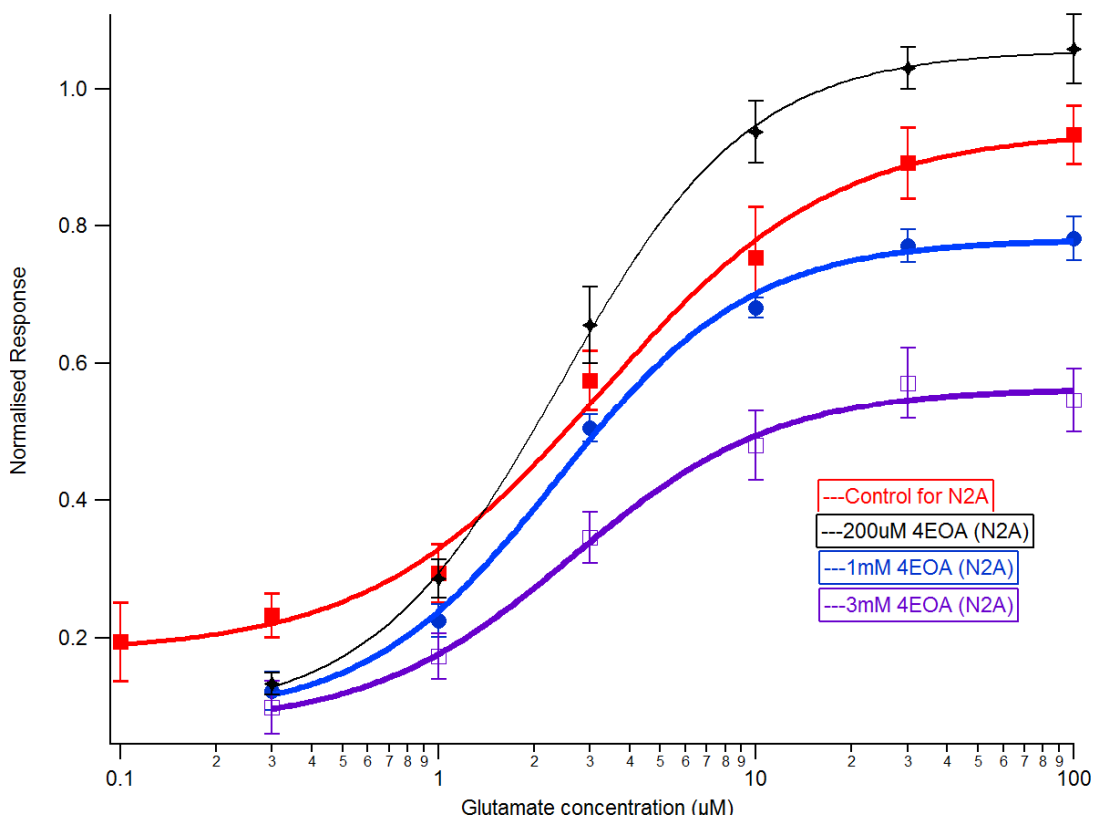


Figure 17. The Effect of Different Concentrations of 4EOA on NMDA Receptor (GluN1/N2A Subunits)

The single dose response curve of 200 μ M, 1mM, and 3mM 4EOA at 0.3 μ M, 1 μ M, 3mM, 10 μ M, 30 μ M, 100 μ M glutamate compared to the wild type GluN2A (control). The error bars were \pm SEM. N=6-10.

There four different concentrations of 4EOA were used to test the inhibitory effect of 4EOA on GluN1/N2A subunits, which were 200 μ M, 1mM and 3mM. The lowest concentration of 4EOA applied was determined by the point which resulted in no change in the normalised response at 100 μ M glutamate, and the highest concentration of 4EOA applied was determined by the solubility of the fatty acid, as the EOA solution could not be used for investigating the inhibition effect when there was a precipitate formation. The range of 4EOA concentration used was much higher than 4BCCA and DA, as the solubility of 4EOA in DMSO solution was higher than 4BCCA and DA, and the 4EOA glutamate dose response curve showed that 3mM 4EOA had a relatively strong inhibitory effect on NMDA receptor (GluN1/N2A subunits).

Concentration of 4EOA (μ M)	Normalised response at 100 μ M glutamate	Rate	EC50 (μ M)
0	0.93310 \pm 0.0429	1.188 \pm 0.237	3.2607 \pm 0.554
200	1.0580 \pm 0.0501	1.461 \pm 0.098	2.418 \pm 0.117
1000	0.78156 \pm 0.0323	1.4376 \pm 0.24	2.3896 \pm 0.292
3000	0.54649 \pm 0.0461	1.3514 \pm 0.333	2.6454 \pm 0.497

Figure 18. The Comparison between Wild Type (GluN2A) and Difference Concentrations of 4EOA on Normalised Response at 100 μ M Glutamate (μ M), Rate and EC50 (\pm SEM)

The 0 μ M 4EOA represented the wild type (GluN2A). 200 μ M 4EOA did not show an inhibitory effect on NMDA receptor (GluN1/N2A) with $p=0.083$. 1mM 4EOA showed an inhibitory effect as the normalised response at 100 μ M glutamate was decreased by 16.2% ($p=0.024$), and 3mM even showed a stronger inhibitory effect as the normalised response was decreased by 41.4% ($p<0.001$). The change in EC50 was not significant for all the 3 concentrations of 4EOA, as $p=0.231$ for 200 μ M 4EOA, $p=0.080$ for 1mM 4EOA and $p=0.811$ for 3mM DA.

Then, as the range for 4EOA concentration used in the experiment was much higher than 4BCCA and DA, which from 200 μ M to 3mM, and therefore a IC50 graph was plot to examine the IC50 value (The main reason for not plotting the IC50 graph for 4BCCA and DA was their small range of concentration). IC50 half maximal inhibitory concentration was the concentration of the drug that was required for 50% inhibition, and it was used to test the effectiveness of the drug's inhibition effect. The glutamate concentration was kept constant at 3 μ M, which was the EC50 value for control glutamate dose response curve.

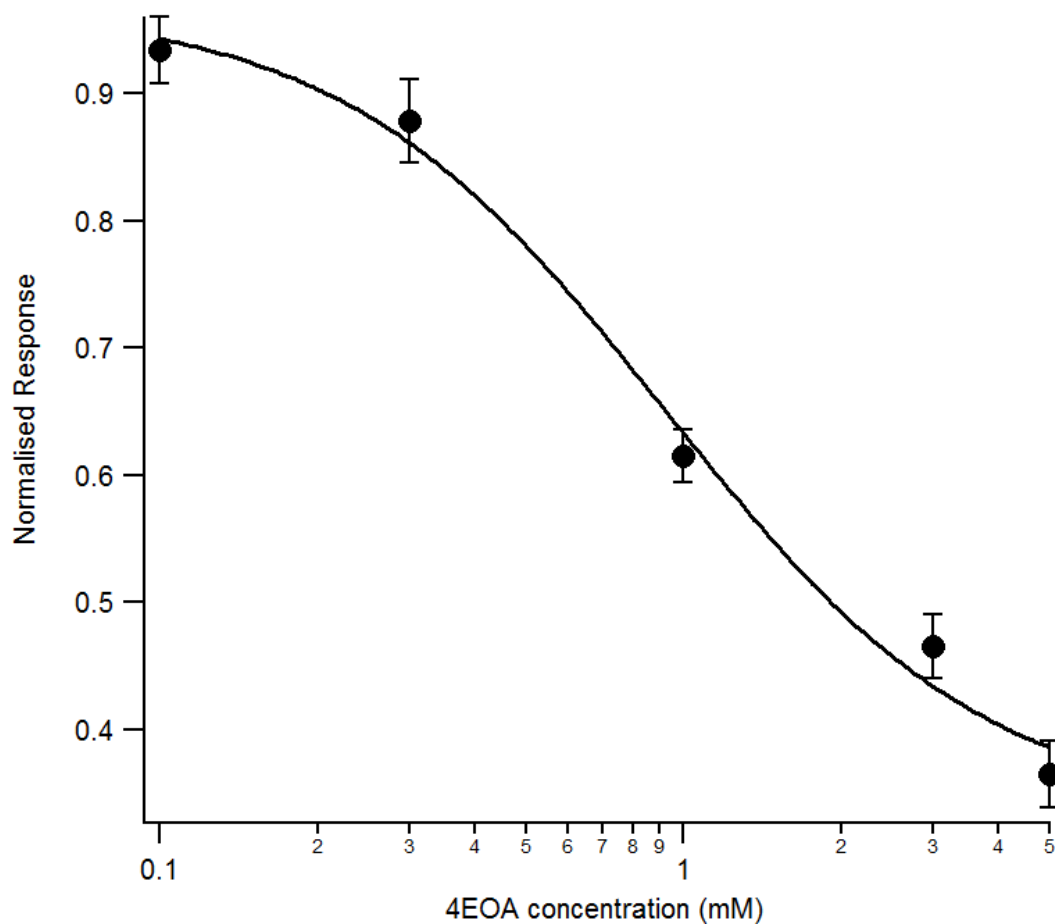


Figure 19. The Inhibitory Dose Response Curve for 4EOA (GluN1/N2A Subunits)

The glycine concentration was kept at 50 μ M. The glutamate concentration was kept at 3 μ M. The 4EOA concentration varied from 100 μ M, 300 μ M, 1mM, 3mM to 5mM. The error bars were \pm SEM. $n=7$. The maximum response was 0.97003 ± 0.123 , the rate was -1.047 ± 0.867 , and the IC₅₀ value was 0.92374 ± 0.322 mM.

Therefore, 0.92374 ± 0.322 mM 4EOA was required to produce 50% inhibition on NMDA receptor (GluN1/GluN2 subunits).

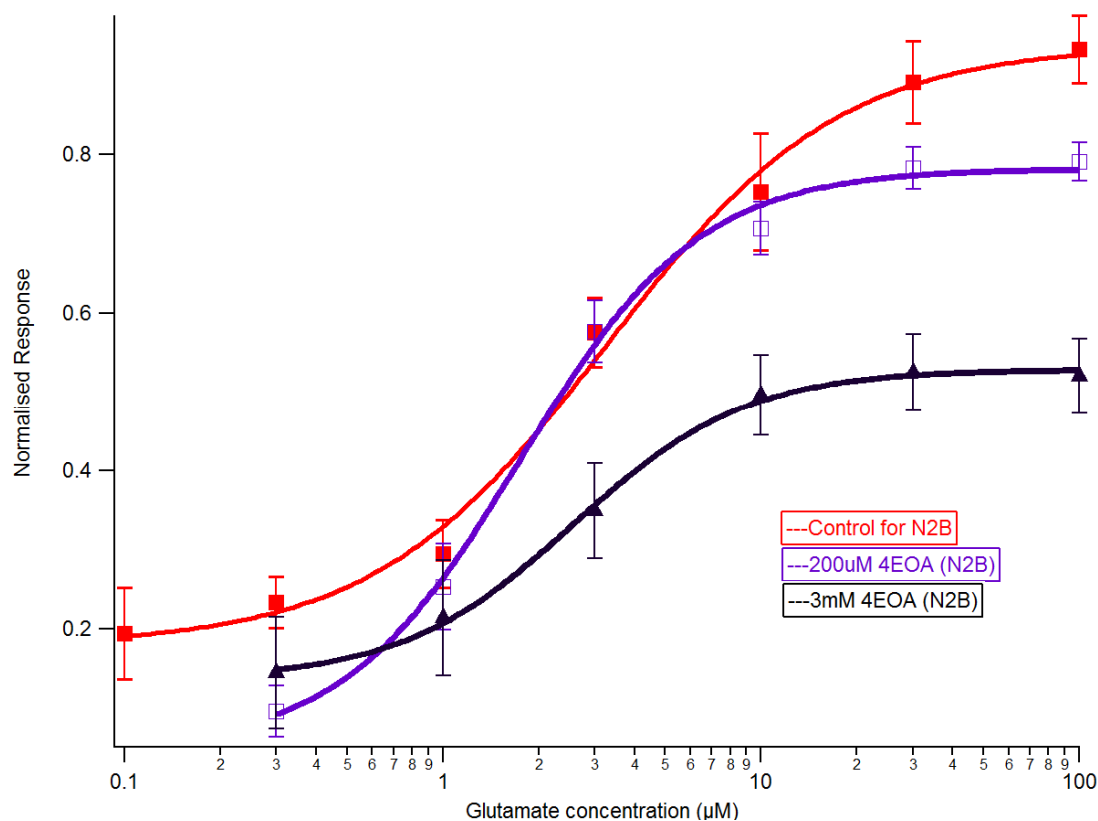


Figure 20. The Effect of Different Concentrations of 4EOA on NMDA Receptor (GluN1/N2B Subunits)

The single dose response curve of 200µM and 3mM 4EOA at 0.3µM, 1µM, 3µM, 10µM, 30µM, 100µM glutamate compared to the wild type GluN2B (control). The error bars were \pm SDE. N=6-10.

Concentration of 4EOA (µM)	Normalised response at 100µM glutamate	Rate	EC50 (µM)
0	1.0050 \pm 0.0047	1.827 \pm 0.147	1.8998 \pm 0.0974
200	0.79176 \pm 0.0239	1.5512 \pm 0.296	1.7561 \pm 0.2
3000	0.51993 \pm 0.0469	1.5989 \pm 0.222	2.5818 \pm 0.231

Figure 21. The Comparison between Wild Type (GluN2B) and difference Concentrations of DA on Normalised Response at 100µM Glutamate (µM), Rate and EC50 (µM) (\pm SDE)

The 0µM DA represented the wild type (GluN2B).

There was an inhibitory effect of both 200µM 4EOA and 3mM 4EOA on NMDAR (GluN2B subunits). The normalised response of 200µM had decreased by 21.2% ($p < 0.01$) compared to the control

(GluN2B). The normalised response of 3mM had decrease by 48.3%, where was a quite strong inhibitory effect ($p < 0.001$). However, both 200 μ M 4EOA and 3mM 4EOA showed no significant change in the EC50 ($p = 0.48$ for 200 μ M 4EOA EC50 and $p = 0.19$ for 3mM 4EOA EC50).

Only the lowest and highest concentration of 4EOA applied on GluN1/N2A, were used to applied on GluN1/GluN2B, which were 200 μ M and 3mM 4EOA respectively, as the main aim of this procedure was to investigate the difference in 4EOA inhibitory effect between GluN1/N2A and GluN1/N2B.

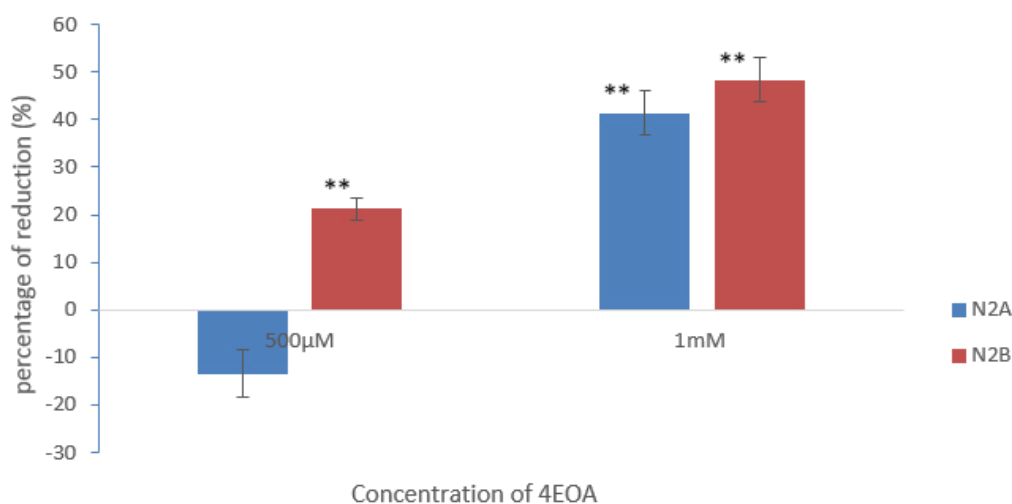


Figure 22. The Comparison of the Percentage of Reduction in Normalised Response for 200 μ M and 3mM 4EOA between GluN1/N2A and GluN1/N2B Subunits

** indicated that $p < 0.05$. The error bars were \pm SDE. N=6-10.

It could be obviously seen that both 200 μ M and 3mM 4EOA exerted an inhibitory effect on N2B, but only 3mM 4EOA showed an inhibitory effect on GluN2A. Also, 3mM 4EOA induced a relatively stronger inhibitory effect on GluN2B (48.3%) compared to GluN2A (41.4%). In addition, there was no significant change of EC50 by all the concentration 4EOA tested on either GluN2A or GluN2B subunits. The main difference was the normalised response. Therefore, GluN2B subunits was more sensitive to 4EOA than GluN2A subunits.

3.4 Comparing the 3 Fatty Acids

All of the 3 fatty acids used in the experiment showed an inhibitory effect on NMDA receptor (both GluN2A and GluN2B subunits) were considered as non-competitive antagonist as they mainly reduce the magnitude of the maximal normalised response but not increase the EC50.

Among the 3 fatty acids, 4EOA was the strongest antagonist as it only stopped to exert an inhibitory effect on NMDA receptor (Glu2A) when the concentration was 200 μ M, and there was still an inhibitory effect on NMDA receptor (Glu2B). 4BCCA had a relatively weaker inhibitory effect, it showed an inhibitory effect on NMDA receptor (GluN2A) when the concentration $\geq 500\mu$ M. DA had

the weakest inhibitory effect on NMDA receptor (GluN2A) as it only showed inhibitory effect when the concentration greater than 750 μ M. Moreover, 4EOA could dissolve in the Ringer's solution when the concentration higher than 3mM, but DA and 4BCCA could only dissolve in the Ringer's solutions when their concentration was 1mM.

GluN2B subunits were more sensitive to the 3 fatty acids used in the experiment than GluN2A subunits, which indicated by a larger percentage of reduction in normalised response found in GluN2B compared to GluN2A.

4. Discussion

4.1 Fatty Acids Act as Non-Competitive Antagonists of NMDA Receptor

NMDA receptor was coupled with calcium channel to form NMDA receptor/calcium channel complexes which were mainly distributed in cerebral cortex, hippocampus and amygdala, and secondarily distributed in midbrain, thalamencephalon and cerebellum (Radley et al., 2007). It had been proved that NMDA receptor had at least 5 pharmacologically independent binding sites: TBS (Transmitter Binding Site), GBS (Glycine Binding Site) and zinc ion binding site, which were located on receptor surface; magnesium ion binding site and PCP (Phencyclidine) binding site, which were located in ion channel. Drug could directly act on these binding sites to influence the function of NMDA receptor (Monaghan, 1989).

Antagonists are drugs that occupy the receptor but do not elicit a response, to prevent the effect of agonists. Competitive antagonists inhibit agonists by competing at the orthosteric site binding site (same binding site), but non-competitive antagonists bind to allosteric binding site (alternative binding site) (Smiley, 2005). All the three fatty acids used in this experiment (4BCCA, DA and 4EOA) are shown to have inhibitory effects on NMDARs were recognised as non-competitive antagonist as they resulted in a decrease in magnitude of maximal response, without increasing EC₅₀. For instance, if the normalised response at 100 μ M glutamate was assumed as the maximal response, 1mM 4BCCA reduced the maximal response by 38.4% for GluN1/N2A subunits, and 41.8% for GluN1/N2B subunits. 1mM reduced maximal response by 13.2% and 35% for GluN1/N2A and GluN1/N2B subunits respectively. 3Mm 4EOA reduced the maximal response by 41.4% and 48.3% for GluN1/N2A and GluN1/N2B subunits respectively. All the glutamate dose response curve of the three fatty acids did not show a significant increase of EC₅₀, as they might work on the allosteric site of the NMDA receptor, and the bond between the active site and the fatty acids used were completely or nearly irreversible (Rogawski, 1992).

The mechanism of DA, 4BCCA and 4EOA inhibiting NMDA receptor was unknown, but they might work on the PCP binding sites, as PCP binding sites are the considered as allosteric binding site where the agonist glutamate do not bind to. With chemical name of N-1-Phenylcyclohexyl-Piperidine (PCP), phencyclidine was a dissociative anesthetic applied clinically the earliest. When it produced anesthetic action, the patient showed mental and behavior disorder, hence it was prohibited from clinical use for a

long time (Morris & Wallach, 2014). However, the mental and behavior disorder caused by it aroused attention of neuroscientist. Anis et al. (1983) firstly stated that PCP and ketamine could selectively reduce neuronal excitability in spine of rats and cats caused by NMDA receptor activation. More widely and deeply research has been performed following Anis's work, to investigate the relationship between action of these drugs and NMDA receptor. Antagonists with stronger action and higher selectivity such as TCP, MK-801 (Di-zocilpine) and its derivative ADC1 were finally synthesized (Kovacic & Somanathan, 2010). The inhibitory effect of these drugs on NMDA-R was non-competitive, large-dose NMDA and glutamate still could not reverse their action, so they were called non-competitive NMDA receptor antagonists (Hillhouse et al., 2015).

Research on radio receptor assays found that the PCP drugs did not influence binding of (^3H -Glu) and (^3H)-CPP, indicating that they did not act on TBS (Theta Burst Stimulation) of NMDA receptor, while NMDA-R agonist CPP, NMDA and glutamate could promote binding of (^3H)-MK-801 or (^3H)-TCP and raise its rate of release, antagonist produced an opposite effect (LePage et al., 2005). Thus, the non-competitive antagonists such as PCP, etc., were bond with PCP binding sites located deeply in ion channel to block calcium channel coupled with NMDA receptor and inhibit calcium ion influx, thereby weakening or antagonizing the effect caused by EAA (Excitatory Amino Acid) (Kovacic & Somanathan, 2010). MK-801 had been tried on treatment of epilepsy, anxiety neurosis and cerebral ischemia, etc., with some curative effects. However, the severe adverse reaction terminated further clinical use of MK-801 (Foster & Fagg, 1987). It was hoped to find out effective drugs to treat CNS disease by binding to PCP sites of NMDA receptor.

Moreover, the non-competitive antagonists of NMDA receptor also commonly acted on the zinc/magnesium binding sites. Many bivalent cations influenced the effect mediated by NMDA receptor to a varying degree, with only Mg^{2+} and Zn^{2+} ion having important physiological significance (Prorok & Castellino, 1998). Inspecting single ion channel recordings from neurons using radio ligand binding experiments proved that Mg^{2+} and Zn^{2+} all could inhibit the excitatory effect regulated by NMDA receptor, yet their mechanism were different: magnesium ion could accelerate dissociation of (^3H)-MK-801 or (^3H)-TCP, while zinc ion had an opposite action. However, the two did not influence binding of (^3H)-CPP with its binding site (Wong, 1991). These results indicated that the binding sites of these two cations were located at different positions: the binding site of magnesium ion was deep in ion channel, while the binding site of zinc ion was on surface of NMDA receptor (Monaghan, 1989).

When the concentration of Mg^{2+} was lower than that of normal extracellular fluid and there was concentration gradient, it could selectively block NMDA receptor. This action was voltage dependent, when the membrane potential was more negative, its blockage action was the strongest and appeared quickly, so it was non-competitive (Nikolaev et al., 2012). Therefore it was argued that at normal resting membrane potential, only a fraction of the reaction caused by low frequency synapse activities was mediated by NMDA receptor activation, and only when membrane potential was at a more negative value ($<-70\text{ mV}$) and NMDA receptor was fully excited to cause relatively long-time reaction

(such as repeated discharging in synapse at high frequency, etc.), Mg^{2+} in extracellular fluid could exert more notable function to hinder it from pathological formation, such as preventing diffusion of foci impulse to peripheral normal tissue, formation of LTP and degeneration of neurons, etc., thereby regulating normal physiological function of NMDA receptor (Ruppersberg et al., 1994). Above actions of Mg^{2+} were also the physiological basis for its anticonvulsion effect.

Zn^{2+} also could selectively block NMDA receptor. Zn^{2+} of low concentration (1~100 nM) could antagonize neuronal excitability in rats caused by NMDA receptor activation, with action appearing fast and being reversible but it was not potential dependent, which were different from magnesium ion (Monaghan, 1989).

The physiological and pathological function of Zn^{2+} in brain are not completely understood yet. Zn^{2+} concentration in hippocampus was the highest, and stimulating neurons in hippocampus by high concentration of K^+ could lead to substantive release of Zn^{2+} , which may participate in regulation and control of excitatory transmission of neurons in hippocampus to exert the function of negative feedback, weaken excitatory reaction caused by EAA and excitatory neurotoxic action (Amico-Ruvio et al., 2011). In contrast, injecting trace of zinc sulfate to hippocampus of rabbits or rats could cause clonic convulsion and neuron necrosis (Pei & Koyama, 1986). These results seemed to show Zn^{2+} at physiological concentration could block NMDA receptor and had inhibitory effect on NMDA receptor, to regulate normal physiological function of NMDA receptor together with Mg^{2+} . When their concentration exceeded physiological concentration, they could also resulted in excitatory effect, or even caused convulsion and neuron necrosis (Noh et al., 2015).

4.2 NMDA Receptor & Epilepsy

In fact, not only AMPA receptor are important in epilepsy, NMDA receptor also plays a role in epilepsy development. The change of NMDA receptor was detected after epilepsy occurrence. The over-expression and over-activation of NMDA receptor may accounted for epilepsy development. Under some pathological conditions, the excessive amount of glutamate in cell can over activate NMDA receptors, then endogenous Mg^{2+} was removed and synchronous depolarization of the postsynaptic membrane of many neurons can take place, which resulted in persistent discharge of the neuron. Eventually, epilepsy developed with epileptic seizures (Ghasemi & Schachter, 2011). On the brain slices of epilepsy animal models and epilepsy patients, the research results for NMDA receptor change after epilepsy were different. Zhu et al. (2004) found that 1 hour after injecting with a sub-convulsive dose (35 mg/kg) Pentetrazol in abdomen, the amount of GluN2A subunit in cortical neuron rose significantly; While 1 hour after injecting with a convulsive dose of (50 mg/kg), the amount of GluN2A and GluN2B subunit rose and then gradually reduced till revived to normal state after 48 hours, but the expression of GluN1 subunit stayed unchanged. However, Auzmendi et al. (2008) stated that injecting convulsant 3-MP (3-mercaptopropionic acid) into rats for four consecutive days leading to recurrent epileptic seizures, by immunohistochemistry and immunoblotting technique, they could observe the expression of GluN2B subunit in dentate gyrus and parahippocampal gyrus

obviously reduced. After injecting 3-MP for 7 consecutive days leading to epilepsy, there was no significant difference in GluN2B subunit expression in such areas comparing with the control group. On brain slices of intractable epilepsy patients resulted from cortical dysplasia, researchers could see significant increase in GluN2B subunit expression by using Western blotting method. Moreover, the number of repeat after-discharge of EFP (Epileptiform Field Potentials) increased in “dysplastic” group (brain slices with cortical dysplasia) compared with the normal brain slices, where EEP was the characteristic expression of later-discharge (Möddel et al., 2005). Such research manifested that the GluN2B expression levels on different epilepsy model were different and the GluN2B expression levels at different phases of the same epilepsy model were also different, which suggested that GluN2B subunit possibly combined with other subunits of NMDA receptor and played a very important role in epileptic activity at different phases (Ghasemi & Schachter, 2011).

The over activated NMDA receptor would lead to a large amount of Ca^{2+} influx, which resulted in calcium overload. As the second messenger of cell signal transduction, Ca^{2+} could activate PKA (protein Kinase A) and PTK (Protein Tyrosine Kinase). Moreover, it bond with CaMK-II (Calmodulin-dependent protein kinase-II), to transduce Ca^{2+} signal to ERK1/ERK2 (Extracellular signal-regulated kinase), where ERK1/ERK2 could be activated under action of some relevant kinase. Then the signal was transduced from cell surface to nucleus and caused cell excitotoxicity. The excitotoxicity of cortical neuron could led to a series of biochemical change in neuron, which would further result in neuron death. (Rogawski, 1992) Meanwhile, the over-activation of NMDA receptor would damage mitochondrial membrane potential, which caused obvious increase in amount of superoxide. Then oxidative stress injury took place in mitochondrion, which finally resulted in neuronal death (Meldrum et al., 1999). Kambe et al. (2008) observed that when hippocampal neurons were exposed to large amount of glutamate, the activity of neuronal mitochondria significantly reduced and the amount of neuron which had immunoactivity also decreased. However, the immunoactivity of cortical neuron and cortical mitochondria were not affected when they were exposed to glutamate. This proved that cortical neuron was less sensitive to cell excitotoxicity caused by the over activation of NMDA receptor. It deduced that the reason for triggering the phenomenon was because of the destruction of mitochondrial membrane potential rather than the intracellular calcium overload. When there were epileptic seizures, the increase of Ca^{2+} would also occur in astrocyte (Rogawski, 1992). On the brain slice model of partial epilepsy caused by 4-AP (4-aminopyridine), the increase of neuronal excitability would induce the release of neurotransmitter, which caused the increase of Ca^{2+} in astrocyte, and the Ca^{2+} mediated cell signal would then cause persistent seizures and consequently epilepsy development. As a positive-feedback mechanism, epileptic attack facilitated the increase of Ca^{2+} in astrocyte and such continuous increase caused continuous seizures. Thus, the cyclic path between neurons and astrocytes was formed (Noebels, 2002). If using Ca^{2+} chelating agent BAPTA to inhibit the increase of Ca^{2+} concentration in glial cell, the epileptic symptoms were relieved (Sun et al., 2009). This indicated that initial seizure and continuous seizure were relevant to the increase of Ca^{2+} in

astrocytes.

Epilepsy consists of a group of highly synchronized brain neurons, and it is a disease caused by self-limiting abnormal discharge and different reasons. The occurrence of epilepsy links to the imbalance of brain excitability and inhibitory neurotransmitter (Sato et al., 1988). Glutamate is the most important excitatory neurotransmitter in the central nervous system, widely distributed in the central nervous system, closely related to epilepsy. Since NMDA receptor is a glutamate receptor, NMDA was over activated when epilepsy developed. Therefore, NMDA receptor antagonists could be used in the treatment of paralysis epilepsy. The NMDA receptor antagonist could be competitive or non-competitive.

In the brain tissue of epilepsy patients and animal models, people could see mossy fiber sprouting phenomenon in hippocampal granule cell (Dudek & Shao, 2004). Chen et al. (2007) found that on chemical kindling and pilocarpine epilepsy model, using selective GluN2A subunit antagonists could inhibit the initiation of epilepsy and mossy fiber sprouting, but the selective GluN2B subunit antagonists did not work on the beginning of epilepsy and mossy fiber sprouting. Meanwhile, both GluN2A subunit antagonists and NR2B subunit antagonists could reduce neuronal death caused by status epilepticus. The traditional NMDA receptor antagonist was nonselective and had obvious side-effects such as causing mental disorder and dyskinesia. These restricted its clinical applications (Ghasemi & Schachter, 2011). The highly efficient GluN2B subunit antagonist could protect CNS from CNS degenerative diseases and brain injuries. Even if it was used with large amounts, it had no side effects of those of the nonselective NMDA receptor antagonists (Higgins et al., 2016). As a result, it was the most widely used clinically.

For chronic epileptic seizure, GluN2B subunit antagonists also had significant advantages over other resistance agents. On brain slice model of chronic epilepsy, Wang and Bausch (2004) compared the impact of three kinds of NMDA receptor antagonist on seizures attacked, mossy fiber sprouting and neuronal survival. They found that GluN2B subunit antagonist, ifenprodil (GluN2B-selective) and its derivatives R025-6981 could reduce epileptic discharges and inhibit mossy fiber sprouting in granulos cells as well as increase neuronal survival of brain slice tissue granular cell layer. The low affinity and non-competitive NMDA receptors antagonist memantine, high affinity and competitive NMDA receptor antagonist D-APV would accelerate cell mossy fiber sprouting and reduce neuronal survival of granular cell and cause epileptic seizures. By long-term D-APV treatment to in vitro hippocampal seizure model, Bausch (2006) found D-APV could reduce neuron excitability, and prevent the subjects from seizures. However, a sodium channel blocker, tetrodotoxin (TTX) would further increase susceptibility to epilepsy. Therefore, plasticity in excitatory synaptic circuits contributed to seizures. Hellier et al. (2009) also found that on chronic in vitro kainic acid epilepsy model, application of non-selective NMDA receptor antagonist SDZ 220-581 could increase frequency of seizure while using R0 25-6981 could cause long term inhibitory effect so as to reduce the frequency of seizure. As a result, for acute and chronic epileptic seizures, GluN2B subunit antagonists could be used as a selective drug,

and had broad clinical application prospects.

4.3 *GluN2B & GluN2A Role in Learning and Memory*

The results indicated that GluN2B was more sensitive to all the 3 fatty acids used than GluN2A, and the difference in the characteristics between the GluN2A and GluN2B may accounted for the difference.

Different NMDA receptors formed by different combinations of GluN1 and GluN2 are provided with different biological characteristics and participate in different physiological and pathological mechanisms. Among them, “GluN2B” was well known as the gene for cleverness for a period of time, although both the NMDA receptor containing subunit GluN2A and GluN2B could show the function of “dual regulation”: can enhance learning and memorizing via Long-Term Potentiation (LTP), also can lead to cognition impairment via Long-Term Depression (LTD) which main symptom is impairment of learning and memorizing (Monaghan, 1989). However, GluN2B produced a stronger effect on regulating intracellular signaling and cell death signaling, and therefore a greater influence on brain function, so it was argued that if GluN2B played a more important role in learning and memory compared to GluN2A (Wyllie et al., 2013). Sun et al. (2000) used RT-PCR technology to detect expression of subunit of NMDA receptor in olfactory bulb, visual cortex and motor area of adult rats to find the subunit GluN2B was mainly distributed in forebrain, especially in cortices. Besides, it was also substantively distributed in hippocampus, thalamencephalon and spinal cord, etc. Its distribution in these regions provided an important structure base for its physiological function.

Currently it is known that GluN2B subunit is composed of 1,456 amino acid residues and its molecular weight is about 170~180 kDa. Extracellularly, GluN2B has N-terminal signal peptide and four sections of transmembrane domain (M1-M4). M2 is a “channel lining” “loop” facing cytoplasm and reflexing toward intramembrane, and serves as the primary structure forming ion channel (Kuner et al., 1996). There is a corresponding aspartic acid residue on M2, thereby forming a loop, which is the domain part of NMDA receptor ion channel hole and controlled the permeability and conductance of this ion channel. Although GluN1 and GluN2 have similar structures (as well as other glutamate receptor subunit such as GluA1-4), GluN2 has a larger CTD (C-Terminal Domain), and contains around 600 amino acids (Maki et al., 2012). Moreover, Martel et al. (2012) stated that CTD of GluN2B promoted excitotoxicity, as the ion channel with GluN2B opens longer when there was an activation of NMDA receptor, which that may resulted in the difference sensitivity to the fatty acids between GluN2A and GluN2B.

When activated, NMDA receptor (GluN2B) is not only permeable to univalent ion Na^+ and K^+ , but is also highly permeable to Ca^{2+} (VanDongen, 2009). Function of NMDA receptor (GluN2B) shows a “dual regulation”, high-frequency stimulation can activate NMDA receptor (GluN2B) to phosphorylate dense area of postsynaptic membrane via calcium/calmodulin dependent protein kinase II to promote formation of LTP (Berberich et al., 2007). Formation of LTP can in turn promote expression of GluN2B subunit to form a benign cycle to enhance learning and memorizing (TSUMOTO, 1992). While after

NMDA receptor (GluN2B) are activated by low-frequency stimulation, LTD can be formed by downstream signaling pathway of non- Ca^{2+} /CaMK-II (Berberich et al., 2007). However, glutamate excitotoxicity may happen when NMDA receptor (GluN2B) are over-expressed or over-activated. The previous viewpoint is that NMDARs inside and outside synapse have different compositions and functions: the intrasynaptic NMDARs are mainly NMDA receptor (GluN2A), which can activate CREB (cAMP response element binding protein) and promote expression of BDNF (brain-derived neurotrophic factor) of CREB target gene to protect nerve. The extrasynaptic NMDARs are mainly NMDA receptor (GluN2B), which can lower membrane potential of mitochondrial membrane and leads to glutamate excitotoxicity (Vizi et al., 2013). Recent research (2013) reported that activation of single intrasynaptic or extrasynaptic NMDARs can protect nerve via signaling cascade of CRE & BDNF, while the glutamate excitotoxicity depends mainly on co-activation degree and duration of both intrasynaptic and extrasynaptic NMDARs. The research also confirmed that both NMDA receptor (GluN2A) and NMDA receptor (GluN2B) participated in the “dual regulation”, and NMDA receptor (GluN2B) had larger effect on intracellular signaling and cell death signaling regulation, yet with mechanism of action was still being controversial (Zhou et al., 2013).

Decrease of GluN2B subunit in both cortical area and hippocampus showed obvious correlation with impairment of learning and memorizing functions (Magnusson, 2010). The rats with GluN2B gene knocked out in hippocampus showed cognition impairment such as spatial or non-spatial amnesia, etc. (Shipton & Paulsen, 2013). NMDA receptor (GluN2B) in cortical area of prefrontal lobe was also important in formation of LTP in cortical area of prefrontal lobe and formation of memory (Wang et al., 2011). When NMDA receptor (GluN2B) in hippocampus and cortices is activated, cation channel near M2 loop will open, extracellular Ca^{2+} flows in, leading to a rise of intracellular Ca^{2+} concentration, finally causing genetic transcription, protein expression and functional change of postsynaptic membrane, thereby establishing LTP, which helps learning, memory and cognition (Berberich et al., 2007).

Among all subunits of NMDA receptor, the mRNA and protein expression level of GluN2B subunit were the most prone to be influenced by age factor (Zamzow et al., 2013). There was an experiment showed that high expression level of GluN2B protein in cortices and hippocampus of rats aged 4 months could promote spatial learning and memory, the GluN2B protein level in forehead cortices and hippocampus for rats aged 26 months dropped dramatically and functions of spatial learning and memory were damaged. This indicated that GluN2B mRNA and GluN2B protein level in cortices and hippocampus showed obvious correlation with age, and also influences spatial learning and memory (von Engelhardt et al., 2008).

For humans, both GluN2A and GluN2B play an important role in the characteristic development of cortex. The GluN2B subunit in the brain is rich in the early postnatal period and the GluN2A subunit gradually increases with the development (Shipton & Paulsen, 2013). A study done by Bannerman et al. (2008) showed that normal Spatial Reference Memory (SRM) in GluN2A-knockout mice was

unaffected but fast-access Spatial Working Memory (SWM) was impaired, whereas subcutaneous mono-injection of ephedrine (30 mg/kg) upregulated GluN2A subunit expression would like to damage SWM. Mathur et al. (2009) studies had shown that systemic administration of selective GluN2B antagonist Ro25-6981 can destroy fear memory in rats aged 3 months, and the effect decreased with age. GluN2B overexpression in mice showed increased learning and memory (White & Youngentob, 2004). These studies on different forms of learning and memory emphasized the importance of GluN2 subunits in learning and memory, and were mainly related to the acquisition of learning and memory.

Cho et al. (2009) further demonstrated that GluN2A knockout rats could not exhibit deprivation-induced depression, but displayed precocious potentiation of the non-deprived eye inputs. This study suggested that compensatory enhancement of the sensory input of the non-deprived eye after monocular vision deprivation is based on a decrease in GluN2A/2B ratio, and the decrease in ratio was achieved by loss of GluN2A subunits. The stimulation frequency of LTP-induced thresholds in GluN2A knockout rats was significantly reduced so that the stimulation frequency (1 Hz) induced by Long-Term Depression (LTD) in wild-type rats was sufficient to induce LTP.

It was hypothesized that synapses with a high ratio of GluN2A/GluN2B could induce the production of LTD by limiting Ca^{2+} entry into the cell, that was, if the ratio of GluN2A/GluN2B increased, more stimulation (such as high frequency stimulation) was required to induce LTP; While more extensive weak stimulation (such as low frequency stimulation) may induce LTD. Conversely, a low GluN2A/GluN2B ratio would decrease the LTP induction threshold, making the weaker response to elevated Ca^{2+} and activating CaMKII to induce LTP levels. This was consistent with the hypothesis that low NR2A/NR2B ratio favored LTP induction. The NMDA receptor subunit, which regulates plasticity-induced effects, has also been studied in other brain regions. Also, the decrease in GluN2A/GluN2B could enhance the strengthening of synapses.

Sensory experience deprivation affects the function of NMDA receptors. For example, after the 24h sleeping time was deprived, the expression of GluN1 subunit in hippocampus was decreased and other NMDA receptor subunits were not affected. But depriving sleeping time for 4h can enhance GluN2A expression (Lebel et al., 2006). Learning and perceived experience could also regulate GluN2B changes. Quinlan et al. (2004) found that after 6d olfactory discrimination task training, GluN2B protein expression levels of rats decreased and GluN2A protein expression of rats did not change, resulting in GluN2A/GluN2B ratio rising. That suggested that piriform cortex NMDA receptors were transported. GluN2A/2B ratio in the brain regions of rats fed in the dark room would like to decrease. Compared with other types of NMDA GluN2, the expression and regulation of NMDA receptor (GluN2B) are more prone to be influenced by external factors, and it delivers a stronger regulating effect on brain function. Thus further research to illuminate the exact action mechanism of NMDA receptor (GluN2B) in cognition functions of learning and memorizing and its action mechanism of “dual regulation” will be of important significance for exploration into prevention and treatment of cognition impairment.

4.4 Clinical Use

Although all of the 3 fatty acids used showed an inhibitory effect on NMDA receptor (both GluN2A and GluN2B). The fatty acids only inhibited the function of NMDA receptor (GluN2A) when the concentration reached a relatively higher level, and no inhibitory effect found when the concentration below 250 μ M for 4BCCA, 750 μ M for DA and 200 μ M for 4EOA. Also, the inhibitory effect on GluN2B became smaller when the concentration of the fatty acids used became lower. So, low dose of the fatty acids may can be used for epilepsy treatment, without causing side effects, although the dose of the fatty acid need to be calculated precisely before put in clinical use and more researches need to be done. The most unexpected finding was that there was such an obvious difference between GluN2A and GluN2B, and more attention needed to focus on the reason resulted in such a difference. Also, as the GluN2B antagonist was always considered as epilepsy treatment, the 3 acids had stronger inhibitory effect on GluN2B may also be good to use as GluN2B antagonist to inhibit epileptic attack.

4.5 Limitations

However, there were some limitations limited the reliability and validity of the results of the experiment. The first was that the fatty acid used not made fresh everytime before the start of one set of experiment. In fact, the fatty acids used for testing GluN2B had been running 1-8 months later than GluN2A test (GluN2A tests done during NOV 2016-May 2017, GluN2B test done during Jun 2017-Aug 2017), in another word, the fatty acids were kept in fridge or freezers for a long period of time. Therefore, there was a debate on whether the quality of the fatty acids changed over time, and was that change in quality indeed affected the difference in the normalised response between GluN2A and GluN2B. Secondly, the solubility of the fatty acids was an obstacle when performing the experiment, as the precipitates forms when the concentration of 4BCCA or DA higher than 1mM, and making the range to plot the IC₅₀ curve very small, and in the end there was no good way found to plot the IC₅₀ curve and get the IC₅₀ value. Also, it was hard to know their inhibitory effect on NMDA receptor if their concentration higher than 1mM. Thirdly, due to the time limitation, only the highest and lowest concentration for 4BCCA, DA, 4EOA tested on GluN2A used for testing GluN2B again to compare the difference between the two subunits (100 μ M and 1mM 4BCCA, 500 μ M and 1mM DA, 200 μ M and 3mM 4EOA used to test their effect on GluN2B), it would be better if try more concentrations, and identified the concentration of the corresponding fatty acid which did not cause inhibitory effect on GluN2B to compare with GluN2A, and even try to plot the IC₅₀ curve for GluN2B. And lastly, this experiment did not show the mechanism of these fatty acids on inhibiting NMDA receptor, and it was hard to speculate the mechanism and put the fatty acids in clinical use.

5. Conclusion

In conclusion, all the 3 fatty acids used in the experiment were non-competitive antagonists, which showed inhibitory effect on NMDA receptor (both GluN2A and GluN2B) when they reached certain concentration, although the mechanism was unknown. 4EOA had the strongest inhibitory effect,

followed by 4BCCA, and DA had the weakest inhibitory effect. The GluN2B subunit is found to be more sensitive than GluN2A subunit to these fatty acids. More researches needed to be done to further examine these fatty acids and put them in clinical use, to help and facilitate epilepsy treatment in the future.

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